CHARACTERISATION OF ACETYLCHOLINE AND ANGIOTENSIN II RECEPTOR MECHANISMS FOR CORTISOL SECRETION IN BOVINE ADRENOCORTICAL ZONA FASCICULATA/RETICULARIS CELLS

by

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This thesis and the work described herein has been produced as a result of work carried out at the University of Edinburgh solely by myself, except where otherwise indicated.

Colin D. Clyne
March 1994

To Mum and Dad

ABSTRACT

Numerous hormones and neurotransmitters have been reported to modulate steroid secretion from the adrenal cortex. In particular, acetylcholine (ACh) and angiotensin II (AII) stimulate both aldosterone and cortisol secretion from the adrenocortical zona glomerulosa (zg) and zona fasciculata/reticularis (zfr) respectively. This study has investigated the cellular mechanisms by which ACh and AII stimulate cortisol secretion from bovine adrenal zfr cells maintained in primary culture.

The ability of ACh and AII to stimulate cortisol secretion from zfr cells was assessed over experimental days 1 (initial isolation of cells) to 5. Freshly isolated cells secreted cortisol in response to ACh and AII. This steroidogenic response was significantly reduced or absent on day 2, and recovered over days 3 and 4, at which time cells were maximally responsive to these agents. The reduced secretory response on day 2 was accompanied by a paradoxical increase in phospholipase C (PLC) activation, indicating that stimulation of PLC by ACh and AII becomes uncoupled from stimulation of steroidogenesis at this time. In contrast, steroidogenic and second messenger responsiveness to adrenocorticotropin (ACTH) and adrenaline, which activate adenylate cyclase, increased over experimental days 1 to 4. This loss of steroidogenic responsiveness to ACh and AII was shown to occur through a defect in the protein kinase C-mediated stimulation of the steroidogenic pathway.

The receptor subtype(s) mediating the steroidogenic responses to ACh and AII were characterised using selective antagonists. Hexahydro-sila-difenidol and p-fluoro-hexahydro-sila-difenidol were potent competitive antagonists of ACh-stimulated cortisol secretion with pA_2 values of 8.68 and 7.96 respectively. Pirenzepine (pA_2= 6.95) and methoctramine (pA_2=6.06) were relatively weak competitive antagonists. These values correlated closely with the established antagonist profile of the M_3 muscarinic receptor. In addition, a rapid desensitisation of this M_3 receptor-mediated response was demonstrated. AII-stimulated cortisol secretion and phosphoinositol production were dose-dependently inhibited both by saralasin and by the AT_1-selective antagonist losartan. PD123177 (AT_2-selective antagonist) was without effect, indicating the presence of the AT_1 receptor. Schild analysis of the inhibition of AII-stimulated cortisol secretion by saralasin and losartan produced pA_2 values of 8.79 and 7.02 respectively. While the pA_2 for saralasin agreed closely with previous measurements in other systems, that for losartan was significantly lower than the values previously reported in other systems, possibly indicating heterogeneity of the AT1 receptor. Indeed, isoelectric focusing of zfr

cell membranes labelled with ^{125}I-AII revealed the presence of two distinct losartan-sensitive AII binding proteins.

Previous studies have shown that in bovine zfr cells, ACh and AII stimulate PLC without affecting adenosine 3',5'-cyclic monophosphate (cAMP) levels. In order to ascertain whether ACh and AII stimulate a common pool of PLC, the effects of these agonists in combination were studied. Partial additivity of response was observed with respect to both cortisol secretion and PLC activation, possibly indicative of heterologous desensitisation. However, evidence was obtained to suggest that in [^{3}H]inositol-prelabelled cells, ACh and AII stimulate incorporation of [^{3}H]inositol into a common hormone-sensitive pool of phosphoinositides, which was distinct from the non-hormone-sensitive pool labelled in the presence of Mn^{2+}.

Finally, changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to ACh and AII were measured in single fura-2 loaded cells by fluorescence microscopy. Both agonists stimulated increases in $[Ca^{2+}]_i$. The Ca^{2+} signals observed in response to ACh were heterogeneous, with 60% of cells showing an initial increase in $[Ca^{2+}]_i$ followed by a decline to a resting level, the magnitude of which was dose-dependent (type 1). The remaining 40% of cells exhibited an initial increase, followed by oscillations in $[Ca^{2+}]_i$, the frequency of which was dose-dependent (type 2). Intracellular Ca^{2+} responses to AII were more homogeneous, and of type 1 only.

These results extend knowledge of ACh and AII effects in inner adrenocortical zone cells. The role of these agonists in the normal and pathological control of adrenocortical function *in vivo* merits further investigation.

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ABBREVIATIONS

AI/II/III	angiotensin I/II/III
ACE	angiotensin converting enzyme
ACh	acetylcholine
ACHE	acetylcholinesterase
ACTH	adrenocorticotropin
ADP	adenosine diphosphate
ADR	adrenaline
AMP	adenosine monophosphate
ANF	atrial natriuretric factor
AVP	arginine vasopressin
BNP	brain natriuretic peptide
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium concentration
(8Br-)cAMP	(8-bromo-) adenosine 3'5'-cyclic monophosphate
CDP-DAG	cytidine diphosphodiacylglycerol
CPSR5	control serum replacement No.5
CRH	corticotropin-releasing hormone
4-DAMP	4-diphenylacetoxyl-N-methyl piperidine methiodide
DHEA(S)	dehydroepiandrosterone(sulphate)
DMSO	dimethylsulphoxide
DOC	deoxycorticosterone
DTT	dithiothreitol
EBS	Earl's balanced salts solution
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(b-aminoethyl ether)N,N,N',N'-tetraacetic acid
fura-2 AM	fura-2 acetoxymethyl ester
$G_i/_s$	inhibitory/stimulatory GTP binding protein
GTP	Guanosine trisphosphate
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
(p-F)HHSD	(parafluoro)hexahydro-sila-difenidol
3βHSD	3β-hydroxysteroid dehydrogenase
Ins	inositol
$InsP_1/_2/_3/_4$	inositol mono/bis/tris/tetrakisphosphate

LDH	lactate dehydrogenase
MKB	modified Kreb's buffer
α-MSH	α-melanocyte stimulating hormone
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)-reduced
NKA/B	neurokinin A/B
NPY	neuropeptide Y
PBS	phosphate buffered saline
PLC	phospholipase C
PKA/C	protein kinase A/C
PMA	phorbol 12-myristate 13-acetate
POMC	pro-opiomelanocortin
PtdIns	phosphatidylinositol
PtdInsP/P_2	phosphatidylinositol mono/bisphosphate
RAS	renin-angiotensin system
TPA	tetradecanoylphorbol 13-acetate
TRIS	tris(hydroxymethyl)aminomethane
VIP	vasoactive intestinal peptide
zf	zona fasciculata
zg	zona glomerulosa
zr	zona reticularis
zfr	zona fasciculata/reticularis

CONTENTS

CHAPTER 1

INTRODUCTION

This chapter presents a general introduction to the adrenal cortex and to the steroidogenic agonists acetylcholine and angiotensin II. The chapter is divided into eight sections: The anatomy and physiology of the adrenal cortex, and the nature of the adrenocortical hormones, are described in sections 1.1 and 1.2. Section 1.3 considers the mechanisms of agonist-stimulated adrenocortical steroidogenesis with respect to the adenylate cyclase and inositol phosphate/calcium signalling systems. The pathophysiology of the adrenal cortex is described briefly in section 1.4. Sections 1.5 and 1.6 introduce the neurotransmitter acetylcholine and the peptide hormone angiotensin II. Each agonist is described in general terms before a more detailed review of their effects on adrenocortical steroidogenesis and second messenger formation. Interactions between acetylcholine, angiotensin II and other agonists are described in section 1.7, and the final section of this introductory chapter describes the aims of this thesis.

1.1 THE ADRENAL GLAND - INTRODUCTION

1.1.1 Historical aspects

The first accurate description of the adrenal glands was by Eustachius in 1563, although his illustrations remained unknown until the early eighteenth century. Thus the first descriptions to receive significant attention were by Caspar Bartholinius the Elder in 1611. Little advancement was made for the next two hundred years, and it was not until 1845 that Huschke noted the distinction between the adrenal cortex and medulla (Huschke, 1845). Ten years later, the critical importance of the gland was emphasised by Addison, who provided detailed descriptions of the symptoms of adrenal insufficiency (Addison, 1855). Brown-Séquard (1856) demonstrated that surgical removal of the adrenals quickly resulted in death, although, as discussed by Neville and O'Hare (1982), this was probably due more to shock and sepsis than to true adrenal insufficiency. Much controversy followed these findings, and it was not until the early 1900s that proof for the adrenal cortex, and not the medulla, being vital to life, was obtained (Wheller & Vincent, 1917; Houssay & Lewis, 1923). The race to isolate an active adrenocortical extract

capable of sustaining adrenalectomised animals quickly followed, and was won by Swingle and Pfiffner (1930). The 1930s also saw the preparation and identification of the major adrenocortical steroids, and in 1948, cortisone was chemically synthesised (Sarett, 1948) and successfully used to treat rheumatoid arthritis the following year (Hench *et al,* 1949). During the next two decades, the relationships between mineralocorticoids and glucocorticoids, their physiology and biochemistry, and the factors controlling their release, were elucidated.

1.1.2 Embryology and development

The human adrenal glands are two ovoid glands bilaterally positioned on the superior pole of each kidney, located in the retroperitoneum. The organs consist of two morphologically and functionally distinct glands, the steroid secreting adrenal cortex, and the inner catecholamine producing medulla, which are of different embryological origin (Crowder, 1957). The cortical tissue is the first to appear, at about the fifth week of gestation in humans, and arises from the mesoderm in the urogenital tract. During the seventh week the mass of the presumptive cortical cells is invaded by cells migrating from the neural crest of the ectoderm, which eventually become surrounded by the mesodermal cells, and form the adrenal medulla. The circulation of the gland is established early, and is continuous with the general circulation by sixty days. During the first trimester, the gland enlarges rapidly such that by the second trimester, it weighs around 4g/kg body weight (35 times the adult value). Most of the cortical volume represents the foetal zone (see section 1.1.4), which rapidly degenerates after parturition, and is completely lost by 1 year, at which time the gland weighs only 50% of the birth weight. The gland enlarges slowly over the next ten years to reach 2g in weight, but doubles in weight during puberty to reach the adult weight at age 18.

1.1.3 Anatomy and blood supply

The paired adult human adrenals weigh about 10 g, and measure approximately 5cm x 2cm. The left adrenal, situated at the tail of the spleen, is crescent shaped, while the more pyramidal right adrenal lies close to the inferior vena cava. Three distinct regions of the human gland are defined: the head, containing mostly medullary tissue, the tail containing mostly cortical tissue and the body containing both tissue types. The cortical tissue accounts for around 80% of the volume and weight of the gland.

The arterial blood supply is derived from three sources: the inferior phrenic artery, the renal artery and the aorta (at the level of the superior mesenteric artery) (Figure 1.1). The

FIGURE 1.1

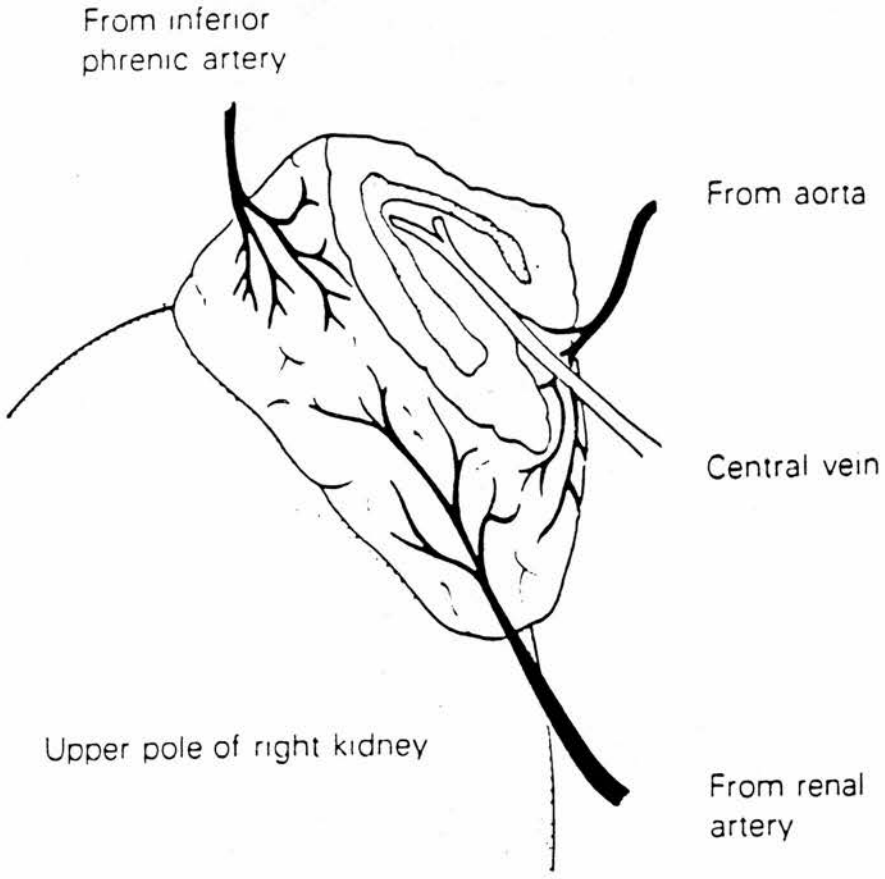

FIGURE 1.1 : Illustration of the human right adrenal gland showing the blood supply and internal structure.

arterial vessels bifurcate en route to the gland, and the adrenal capsule is thus penetrated by up to 50 or 60 arterial twigs, which form the subcapsular plexus. Effluent blood is collected into a single central vein emerging from the anterior surface of the gland. The right adrenal vein passes directly into the superior vena cava while the left joins the inferior phrenic vein, before entering the left renal vein.

The intra-adrenal vascular architecture has been described by Dobbie *et al* (1968). Arterial blood flows from the subcapsular plexus into parallel capillaries which descend through the zona fasciculata (see section 1.1.4) before reaching the zona reticularis, where they form the plexus reticularis. Blood either drains from this plexus into the sinusoids of the alar raphe, or, in regions of the gland containing medullary tissue, continues towards the medulla. This cortico-medullary portal system is thought to be the sole source of medullary blood in the rat and bovine adrenal (Vinson *et al,* 1985), although the human medulla is also supplied directly by the arteria medullae (Flint, 1900). The deeper cortical layers thus have no direct arterial supply, but receive blood which has already perfused the outer cell layers. This centripetal arrangement is extremely well preserved between species, and is found even in the largest mammals such as the elephant (Krumley & Buss, 1969) and whale (Race & Wu, 1961). Thus, in order to maintain adequate perfusion of the deeper cortical layers in larger mammals, adrenal bulk is increased by lateral expansion and convolution, rather than increases in adrenocortical width. The centripetal blood supply has important implications in the gradient theory of adrenocortical zonation (see section 1.1.4).

1.1.4 Adrenocortical histology and zonation

The adrenal gland is enclosed by several layers of elongated connective cells surrounded by collagen fibres, which form the capsule. The remainder of the cortex consists of three distinct cell types arranged in concentric zones, the zonae glomerulosa, fasciculata and reticularis (Figure 1.2). Directly underneath the capsule, and forming the periphery of the cortex, lies the zona glomerulosa (zg). Accounting for around 5% of the total mass of the cortex, the relatively small cells of the zg are arranged in rounded clusters (glomus = ball), with a high nuclear/cytoplasmic ratio. The cytoplasm contains comparatively little lipid, and is extremely rich in elongated mitochondria. The majority of the mass of the cortex (70%) is composed of the zona fasciculata (zf). The large zf cells are arranged in columnar layers (fascis = bundle), and are characterised by their prominent plasma membranes, low nuclear/cytoplasmic ratio, extensive lipid droplets and small, ovoid mitochondria. The remaining 20% of cortical tissue comprises the innermost zona reticularis (zr); these cells are of intermediate size, lipid content and cytoplasmic volume.

FIGURE 1.2

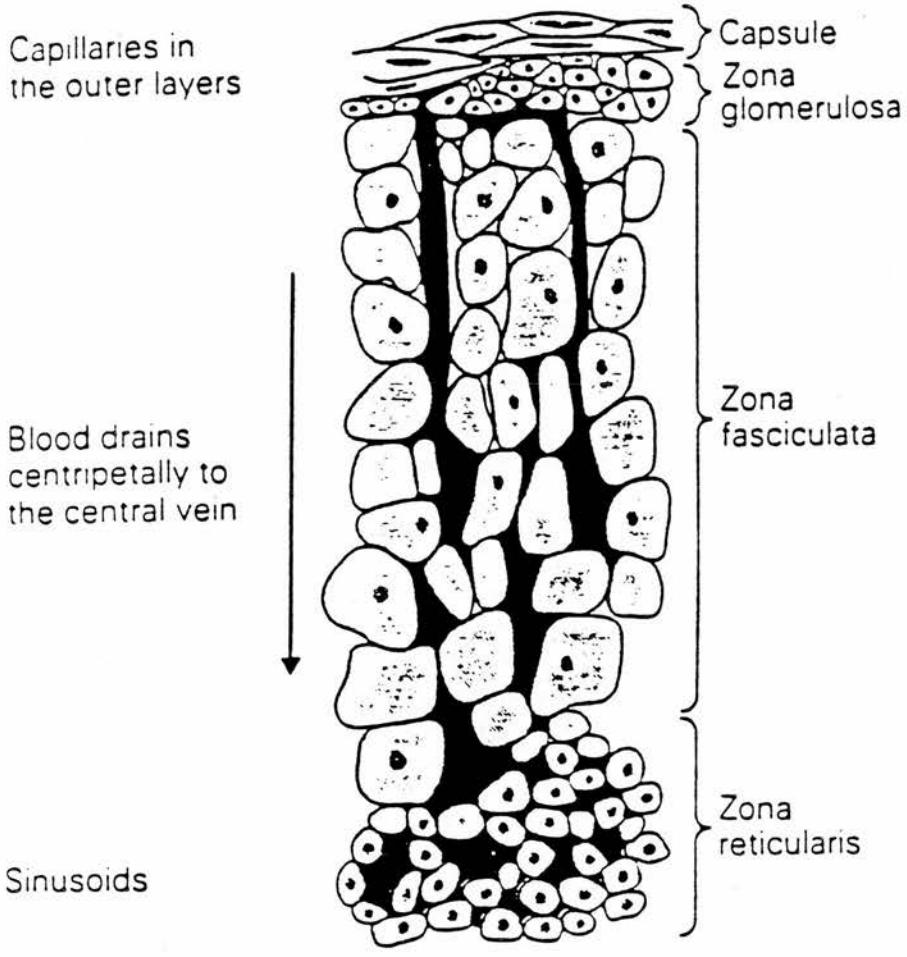

FIGURE 1.2 : Zonation of the mammalian adrenal cortex. Diagramatic representation of the arrangement of cells in a cross-section of the adrenal gland.

Exhibiting a network-like arrangement (rete = net), zr cells are usually described as "compact". The cells of the zr are more pigmented than those of the other adrenocortical zones, due to the presence of cytoplasmic lipofuscin granules.

The exact cellular arrangement of the cortex varies between species. In the human gland, there is no sharp delineation between the zg and the zf, and the zones merge imperceptibly, with zf cells often observed directly underneath the capsule. In contrast, the zg of the bovine cortex is sharply demarked from the inner zones, while the zf and zr form a single uniform layer. The three zones are clearly distinguishable in the rat, guinea-pig and rabbit cortex, while the amphibian adrenal cortex (interrenal gland) displays no significant zonation.

Further adrenocortical zones have been described, with species and developmental variation. In the male mouse, the zr is replaced by the so-called X-zone (Dunn, 1970; Shire, 1979), which disappears following sexual maturity. Rat, rabbit, cat and dog adrenal cortices exhibit a fourth zone, situated between the zg and the zf, termed the zona intermedia (zi) (Deane, 1962). The human and primate foetal adrenal cortex exhibits only two zones, the outer definitive and the inner foetal zone. The foetal zone, first described by Elliot and Armour (1911), enlarges rapidly during gestation, and represents the bulk of the foetal cortical mass before its degeneration following parturition.

The division of the adrenal cortex into the zg, zf and zr was first proposed over one hundred years ago (Arnold, 1866). Although this distinction was made on histological and anatomical grounds, it is now clear that the adrenocortical zones are also functionally distinct. Differences in the enzyme complement of the zones lead to differences in the steroids secreted by them. Thus, aldosterone is secreted exclusively by the zg, while cortisol is produced solely by the inner zfr. Androgens are secreted principally by the zr (see section 1.2).

It has been proposed that the functional zonation of the cortex arises as a result of the blood supply to the organ. As discussed in the preceding section, the adrenocortical blood supply is centripetal in nature, and the adrenocortical sinusoids are arranged so that blood tends to pool in the innermost zone. As cells of the inner zones are exposed to the secretory products of cells nearer the capsule, a steroid gradient is thought to exist between the capsule and the medulla (reviewed in Hornsby, 1987). It is proposed that the steroidogenic enzymes can be regulated by steroids, allowing the activation or inhibition of a given cellular enzyme depending on its position in the steroid gradient. Thus, the

dominant enzymatic activities of the steroidogenic cell would be determined by its position in the cortex.

Until relatively recently it was assumed that the anatomical separation of the adrenal cortex and medulla, coupled with the centripetal blood supply to the gland, would preclude any functional contact between medullary and cortical cells. However, in the rat adrenal gland, projections of chromaffin cells from the medulla towards the outer adrenocortical zones have been observed (Gallo-Payet *et al,* 1987). A similar invasion of medullary tissue into cortical regions appears to exist in the porcine adrenal, which also showed invasion of the medulla by cortical cells (Bornstein *et al,* 1991). This cortical invasion of the medulla was also seen in the bovine adrenal. In porcine and rat adrenals, chromaffin cells within the cortex appear to form intimate connections with cortical cells (Bornstein *et al,* 1991).

1.1.5 Adrenocortical Innervation

It is well established that the adrenal medulla receives a rich preganglionic sympathetic cholinergic innervation. Fibres arising from the splanchnic nerve appear to penetrate the adrenal cortex without synapsing and terminate directly on the medullary chromaffin cells (Symington, 1969). It has widely been assumed that in contrast to the medulla, the adrenal cortex receives no innervation. However, recent studies have provided conclusive evidence for the presence of adrenocortical nerve fibres in many species. The subject of adrenocortical innervation is reviewed in detail in Charlton (1989), on which the following brief discussion is mainly based.

Robinson (1977) described a complex innervation of the ovine adrenal cortex. Acetylcholinesterase (AChE)-positive nerve fibres were distributed in the cortical tissue and formed a distinct plexus in the zr. The outer zones of the cortex also exhibited aminergic nerve fibres, as determined by catecholamine-fluorescence histochemistry. A similar anatomical distribution of nerve fibres has been described in rat, pig and mouse adrenal cortex (Unsicker, 1971; Migally, 1979). More recently, Charlton *et al* (1991) have used AChE-histochemistry to demonstrate a complex cholinergic innervation, possibly derived from the splanchnic nerve, in normal human adrenocortical tissue. A subcapsular nerve plexus was evident in some specimens, and nerve trunks extended through the zf before forming a second plexus in the zr. The distribution of fibres was consistent with innervation of either cortical cells and/or blood vessels. The human adrenal cortex has also been shown to contain noradrenergic (dopamine β-hydroxylase-positive) nerve fibres, showing a similar distribution to the cholinergic innervation

(Gilchrist *et al,* 1993). With the exception of the innermost zr, noradrenergic neurones were present throughout the cortex, and were found in juxtaposition to both blood vessels and steroidogenic cells (Charlton *et al,* 1992).

Several studies have demonstrated increased steroidogenesis in response to electrical stimulation of the splanchnic nerve (Charlton, 1989, and section 1.5.3), although it is unclear whether this represents a direct effect on the steroidogenic cells themselves or an indirect effect related to changes in adrenocortical blood flow. Although many putative adrenocortical neurotransmitters directly stimulate steroid secretion from isolated adrenocortical cells (see Tables 1.2 and 1.3), stimulation of the splanchnic nerve is known to result in a decrease in adrenocortical vascular resistance (Engeland *et al,* 1985; Edwards *et al,* 1986; Engeland & Gann, 1989). The resultant increase in adrenocortical blood flow could stimulate steroidogenesis by increasing the delivery of humoral stimulants such as ACTH, O_2 or steroid substrates to the gland. Furthermore, the rate of steroid secretion by the isolated perfused rat adrenal gland can be markedly increased by increasing the rate of delivery of perfusion medium to the gland (Hinson *et al,* 1986). The relative importance of neural effects on adrenocortical blood vessels and direct effects on adrenocortical cells remains to be established.

1.1.6 Experimental adrenocortical preparations

Many of the pioneering studies on adrenocortical function were performed using intact animals *in vivo*. While it is clearly desirable to study the adrenal cortex in its natural environment, the measurement of steroid hormones in body fluids gives little or no information about the source of the measured hormones, or the extent to which they have been metabolised. Furthermore, the concentration of adrenal hormones in plasma is extremely low, and problems of assay sensitivity led to the development of *in situ* methods of measuring secretion from the whole organ. This technique of catheterisation of the adrenal vein allows the measurement of adrenal hormones in the effluent blood (at much higher concentrations than in peripheral plasma) while maintaining the organ in its natural environment.

These *in vivo* methods do not allow the contribution of each individual adrenal cell type to be assessed, nor the effect of cortico-medullary interactions to be analysed. They are also unsuitable preparations with which to investigate the cellular mechanisms of agonist-stimulated steroidogenesis. Thus, the need for purified preparations was recognised, and many workers used adrenal tissue slices incubated in simple salt solutions *in vitro*. Although crude preparations of zg and zfr slices can be prepared in this way, tissue slices

suffer from poor oxygenation and impaired delivery of test substances to inner cell layers, resulting in reduced steroidogenic responsiveness compared to results of *in vivo* and *in situ* studies.

These problems are overcome by the culture of adrenocortical cells. Following enzymatic digestion of tissue slices, dispersed cells can be rigourously purified by density gradient centrifugation or column filtration and maintained in chemically defined media. Delivery of oxygen and test substances is enhanced, and secreted steroids can accumulate at high concentration. Furthermore, the pooling of tissue from many different animals to produce a composite cell preparation reduces the inherent biological variability associated with whole animal studies. Purified cells from each zone can be further disrupted to produce subcellular organelle fractions for enzymatic studies, or plasma membrane fractions for hormone receptor studies.

1.2 THE ADRENOCORTICAL STEROIDS

Over 40 different steroids have been isolated from the adrenal cortex, although many of these represent intermediates or precursors of the major secretory products. The main adrenal steroids are those with mineralocorticoid or glucocorticoid activity, although some sex steroids (androgens, estrogens and progestins) are also secreted. All steroids have a common ring structure consisting of three six-membered rings and one five-membered ring (Figure 1.3), and their biological activity is determined by the number and structure of the functional groups attached to this ring nucleus.

Early studies using capsular (95% zg) and decapsulated (zfr) portions of rat and bovine adrenal cortex established that aldosterone, the major mineralocorticoid in all species, is produced exclusively in the zg, while cortisol, the major glucocorticoid in human, bovine, sheep and guinea-pig, is produced solely in the zfr. Corticosterone (the major secretory product in rat, rabbit and mouse) is produced both by the zg and the zfr. Although the zf and zr secrete the same steroids qualitatively, sex steroids such as dehydroepiandrosterone appear to be produced preferentially by the zr. The evidence which established this functional zonation is reviewed in Tait *et al* (1980a).

1.2.1 Mineralocorticoids and glucocorticoids

The major endogenous mineralocorticoid is aldosterone, although corticosterone and 11-deoxycorticosterone also exhibit weak mineralocorticoid activity (Table 1.1). The mineralocorticoids are characterised by the presence of a hydroxyl group at carbon-11

FIGURE 1.3

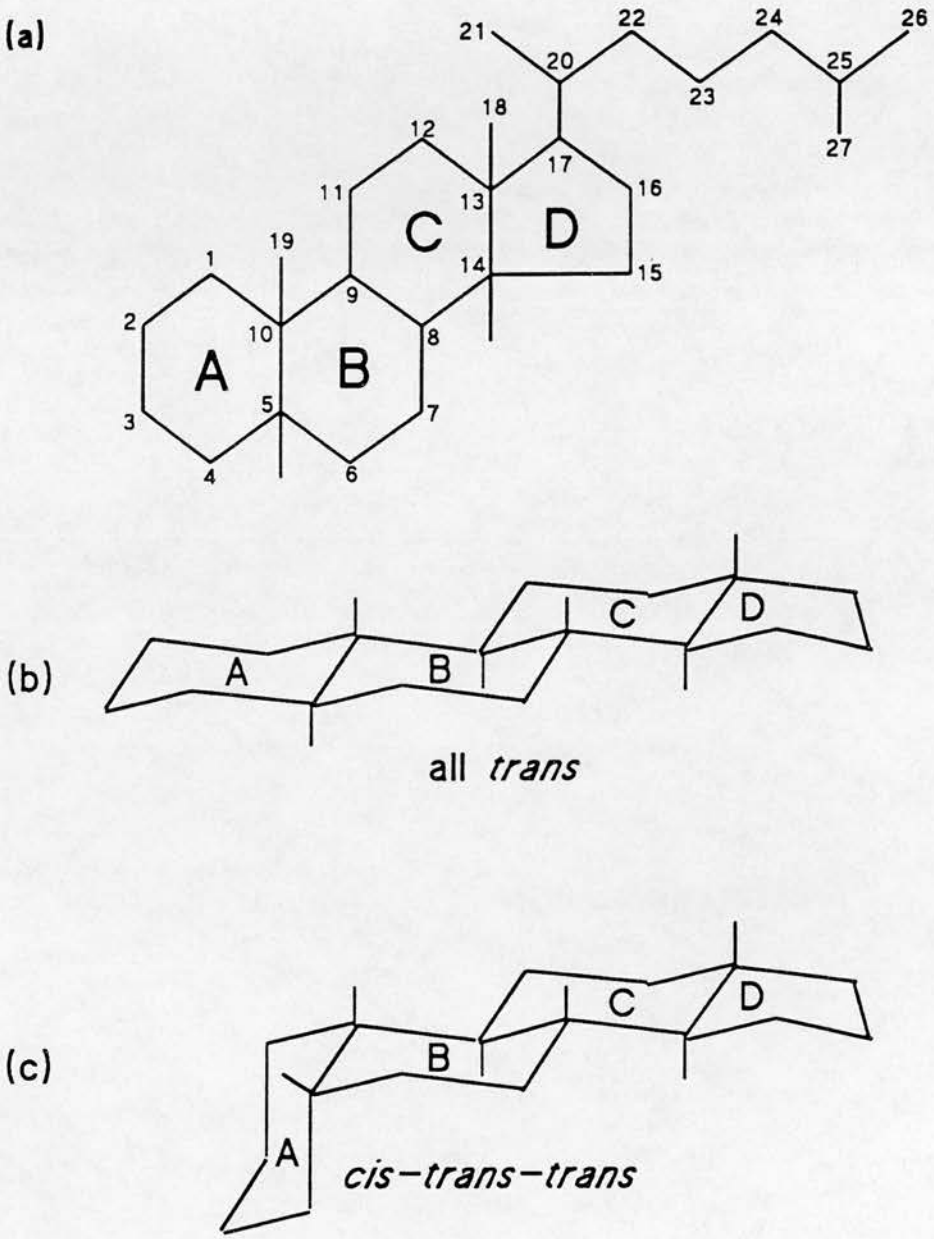

FIGURE 1.3 : Structure of the adrenocortical steroids. The nomenclature of the ring nuclei and numbering of the carbon atoms is shown in (a). Rings B:C and C:D are invariably joined in the *trans* configuration, while rings A:B can be either *trans* or *cis*. Thus A:B *trans* compounds exhibit a planar structure (b), while in A:B *cis* compounds, the A ring lies in a second plane at approximately 90° to the B:C:D rings (c).

TABLE 1.1

		relative biological potency		
steroid	*secretion rate**	*gluco**	*mineral**	*androgen***
cortisol	100	100	100	-
corticosterone	5	30	160	-
aldosterone	0.75	-	30000-50000	-
DOC	1.2	-	1000	-
androstendione	3	-	-	20
DHEA.S	125	-	-	10
DHEA	8	-	-	10
testosterone	0.06	-	-	100
dexamethasone (syn)	-	3700	-	-
fludrocortisone (syn)	-	1000-2000	15000	-

* relative to cortisol
** relative to testosterone

TABLE 1.1 : Secretion rates and biological activities of the major steroids secreted by the human adrenal cortex. Secretion rates are given relative to that of cortisol (around 15 - 30 mg per day in humans), and biological activities expressed relative to cortisol or testosterone. For comparison, the activities of two synthetic (syn) steroids are given. Abbreviations: DOC, deoxycorticosterone; DHEA(S), dehydroepiandrosterone(sulphate).

and an aldehyde group at carbon-18. Released under conditions of sodium depletion, decreased blood volume and blood pressure, the physiological function of aldosterone is to conserve salt by directly stimulating the absorption of sodium in exchange for the excretion of K^+ or H^+ by the renal distal tubules.

The mechanism by which aldosterone stimulates sodium absorption was originally elucidated through studies on the isolated toad bladder, a convenient *in vitro* model for measuring ion transport across epithelial cells (Fraser, 1971; Edelman & Fanestil, 1970). More recent work has utilised cultured epithelial cells (Handler, 1983). The effect of aldosterone on ion transport is characterised by a latent peroid of around 1 h, followed by an early response during which sodium transport is stimulated and transepithelial resistance is decreased. This is followed by a late response (after around six hours) in which continued sodium transport is accompanied by a constant stable transepithelial resistance (Garty, 1986). Aldosterone binds to a specific high affinity cytosolic receptor (section 1.2.2) which translocates to the cell nucleus on agonist binding and activates or represses specific genes by interacting with hormone-responsive elements in the DNA. Protein synthesis is then stimulated, the importance of which is indicated by the inhibitory effects of actinomycin D on aldosterone-stimulated ion transport in the mammalian kidney (Horisberger & Diezi, 1984). A rapid increase in transcription of the α and β subunit genes of the Na^+/K^+-ATPase has been observed in response to aldosterone (Verry, 1990), although at present the identities of the other proteins involved in the action of aldosterone are unknown. Possible candidates include citrate synthase, sodium permease enzymes, or protein kinases/phosphatases which may modulate the activity of membrane ion channels (Reviewed in Horisberger & Rossier, 1992).

Glucocorticoids are characterised by the presence or absence of hydroxyl groups at C-11 and C-17. The main naturally-occurring glucocorticoids are cortisol and corticosterone (Table 1.1). In contrast to the relatively specific actions of aldosterone, the glucocorticoids exert a wide variety of metabolic and non-metabolic effects (reviewed in David *et al*, 1970). Glucocorticoid effects on metabolism are generally antagonistic to those of insulin ie. mobilisation of lipid, deposition of glycogen, inhibition of protein synthesis in peripheral tissues and stimulation of hepatic gluconeogenesis and protein synthesis. In addition, glucocorticoids act to suppress allergic and inflammatory reactions, decrease resistance to infection and suppress lymphoid function. The mode of action of the glucocorticoids appears to be similar to that of other steroid hormones, ie. induction of nuclear protein synthesis following interaction of glucocorticoids with specific cytosolic receptors (O'Malley *et al*, 1991).

1.2.2 Mineralocorticoid and glucocorticoid receptors

Corticosteroid receptors have been classsified according to the differential binding affinity of aldosterone. Those receptors exhibiting high affinity for aldosterone (ca.10^{-9} M) are found in most aldosterone target tissues and designated type 1 receptors, while lower affinity (ca. 5 x 10^{-8}M) receptors are classed as type II. The type 1 receptor has been cloned, and shown to be a 107 kD protein with a C-terminal steroid binding region and a 70 amino acid section corresponding to the DNA binding region (Arriza *et al,* 1987; Evans, 1988). Both type I and type II receptors show little selectivity between aldosterone and cortisol (Sheppard & Funder, 1987), and, since circulating levels of cortisol greatly exceed those of aldosterone, a great deal of research has focused on the mechanism by which mineralcorticoids can exert their effects. It has recently been shown that aldosterone target tissues contain higher levels of 11β-hydroxy steroid dehydrogenase (11β-HSD) activity than other tissues. 11β-HSD converts cortisol into the relatively inactive compound cortisone, thus allowing intracellular steroid receptors to bind aldosterone rather than be saturated with cortisol (reviewed in Stewart *et al,* 1987; Funder *et al,* 1988).

1.2.3 Adrenocortical steroid biosynthesis

The precursor of all adrenocortical steroids is cholesterol, stored in adrenocortical lipid droplets as cholesterol ester. Human adrenocortical cells, although capable of *de novo* cholesterol synthesis from acetate (Hechter *et al,* 1953) derive the majority of their cholesterol from plasma low density lipoproteins (LDL) (Brown *et al,* 1979), which are internalised by receptor-mediated endocytosis. In contrast, the major source of cholesterol for rat adrenocortical cells is high density lipoproteins (HDL). Free cholesterol is made available for steroidogenesis by the action of cholesterol esterase, while cholesterol ester synthetase is responsible for the storage of cholesterol as cholesterol ester.

The understanding of steroid synthetic mechanisms has changed markedly over the last few years. It was previously assumed that a large number of separate enzymes with strict substrate specificity were necessary for the various reactions and interconversions known to occur in adrenocortical cells. However, as the steroidogenic enzymes were cloned, it became clear that comparatively few enzymes, each capable of catalysing more than one activity, are involved in the conversion of cholesterol to active steroid hormones. Most of the steroidogenic enzymes are members of the cytochrome P450 family of oxidases, which reduce atmospheric O_2 with electrons from NADPH (reviewed in Miller, 1988) :

$$NADPH + H^+ + O_2 + RH \longrightarrow R\text{-}OH + H_2O + NADP^+$$

Four distinct P450 enzymes have been shown to be involved in adrenocortical steroidogenesis: (i) P450scc (side-chain cleavage) mediates the conversion of cholesterol to pregnenolone, a reaction involving three separate activities (20α-hydroxylation, 22-hydroxylation and cleavage of the cholesterol side-chain between C20 and C22) (see Figure 1.4). (ii) P450c11 mediates 11-hydroxylase, 18-hydroxylase and 18-methyloxidase activities. (iii) P450c17 mediates 17α-hydroxylase and 17,20-lyase activities, while (iv) P450arom (aromatase), more commonly found in the gonads, but also present in the adrenal, mediates aromatisation of androgens to estrogens. In addition, pregnenolone is converted to progesterone by one or more enzymes with 3β-hydroxysteroid dehydrogenase and $\Delta^5 \longrightarrow \Delta^4$ isomerase activity, while a 17-keto reductase mediates the conversion of androstendione to testosterone. P450scc and P450c11 are mitochondrial enzymes, while P450c17 and P450arom are located in the endoplasmic reticulum.

The first, and rate-limiting step in the synthesis of the steroid hormones is the conversion of cholesterol to pregnenolone by P450scc (Figure 1.4). Each step in the reaction sequence requires reducing equivalents from NADPH, and these are supplied by a mitochondrial electron transport system. Electrons from NADPH are accepted by adrenodoxin reductase, a membrane-bound flavoprotein, and transferred to P450scc via adrenodoxin, a soluble iron/sulphur protein of the mitochondrial matrix (reviewed in Miller, 1988). The conversion of cholesterol to pregnenolone is the major hormonally-regulated step of the steroidogenic pathway (see section 1.3).

P450c17 exhibits both 17α-hydroxylase and 17,20 lyase activities, formerly attributed to two separate enzymes. Pregnenolone, once formed from cholesterol, can therefore enter either mineralocorticoid, glucocorticoid or androgen synthetic pathways, depending on the activity of P450c17 (see Figure 1.5). If neither hydroxylase nor lyase activity is present, pregnenolone is converted to progesterone and then ultimately to aldosterone. If hydroxylase activity alone is present, P450c17 converts pregnenolone and progesterone to 17α-hydroxypregnenolone and 17α-hydroxyprogesterone respectively, which can be converted to cortisol via 11-deoxycortisol. The 17α-hydroxylated steroids can however be further metabolised by the 17,20-lyase activity of P450c17 to dehydroepiandrosterone (DHEA) and androsterone respectively, the precursors of testosterone and estradiol.

The factors determining which activity (hydroxylation or lyase) P450c17 performs remain obscure. P450c17 is associated with the endoplasmic reticulum membrane where

FIGURE 1.4

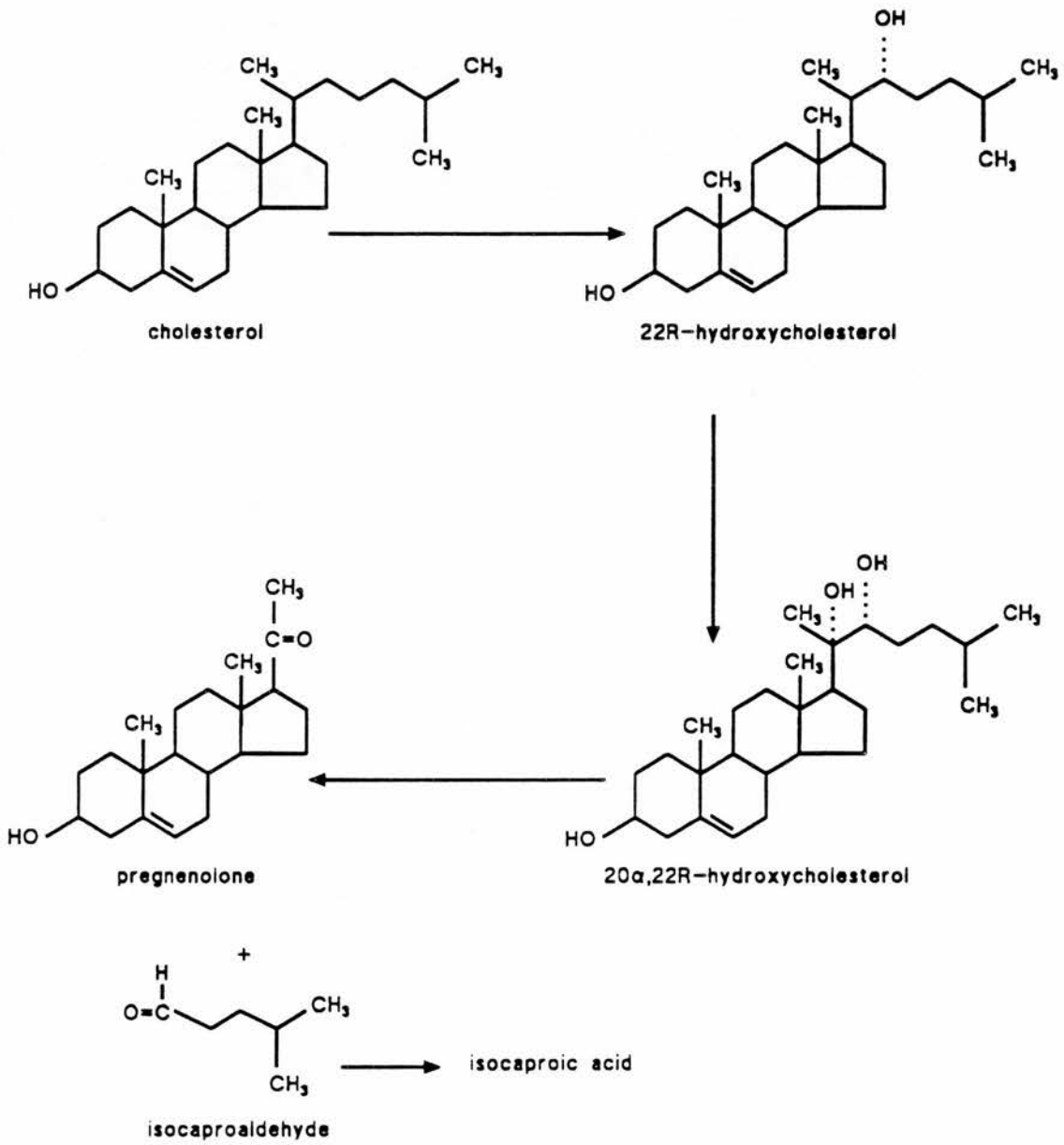

FIGURE 1.4 : Reaction sequence for the conversion of cholesterol to pregnenolone by P450scc.

FIGURE 1.5

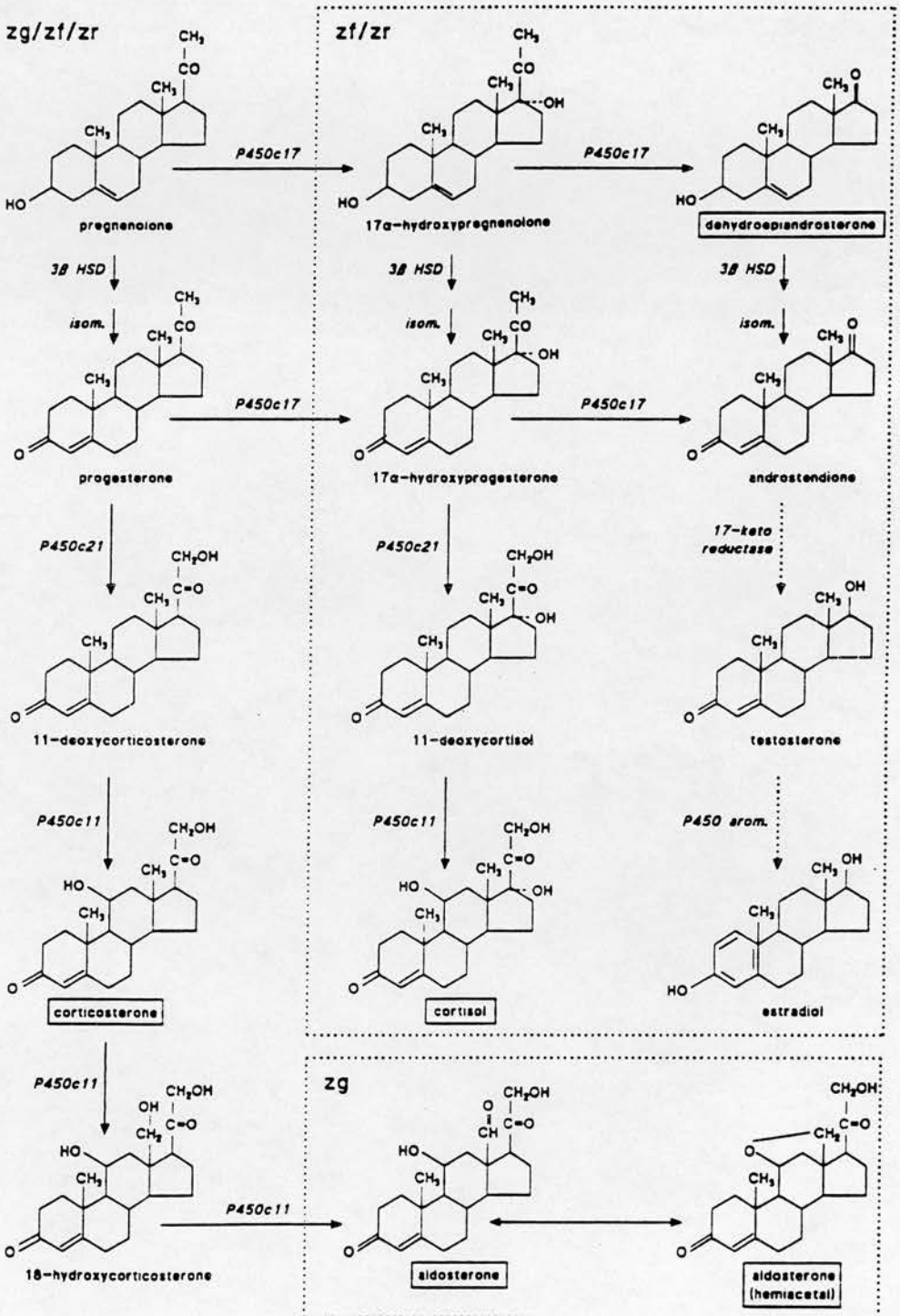

FIGURE 1.5 : Simplified diagram showing the major steroid interconversions occurring in the mammalian adrenal cortex. Reactions confined to particular adrenocortical zones are enclosed in dotted boxes. The principal secretion products are indicated by solid boxes. Abbreviations: 3β HSD, 3β hydroxysteroid dehydrogenase; isom., isomerase.

it receives reducing equivalents from P450 reductase and cytochrome b_5 (Kominami *et al*, 1980; Tamburini & Gibson, 1983). The availability of electrons to P450c17 appears to influence its activity. The ratio of lyase/hydroxylase activity can be increased by increasing the ratio of P450 reductase to P450c17 *in vitro* (Onoda & Hall, 1982). Furthermore, the association of P450c17 with P450 reductase and cytochrome b_5 can be influenced by phospholipids and other small molecules (Muller-Enoch *et al*, 1984), and this may represent a mechanism whereby intracellular messengers could determine the dominant enzymatic activities of the cell.

The major adrenocortical steroids cortisol and aldosterone are synthesised from 11-deoxycortisol and corticosterone respectively by the action of a single enzyme, cytochrome P450c11. This raises the question as to how aldosterone and cortisol secretion can be confined to the zg and zfr respectively, since previous studies had suggested the presence in the zg of a unique aldosterone-synthesising enzyme (Meuli & Muller, 1983,1984). In the rat adrenal cortex, P450c11 is now known to exist in two different isoforms, both of which have been cloned and shown to be the products of different genes (CYP11B1 and CYP11B2) (Nonaka *et al*, 1989; Matsukawa *et al*, 1990). Both isoforms show 11β- and 18-hydroxylase activties, although only one form (CYP11B2) exhibits aldosterone synthetase (methyl oxidase) activity. The gene transcript of this isoform is present exclusively in the zg, while that of the non-aldosterone synthesising isoform (CYP11B1) is present throughout the adrenal cortex (Yabu *et al*, 1991). Furthermore, mRNA levels of the two isoforms are subject to differential regulation by factors known to selectively influence cortisol and aldosterone secretion such as dexamethasone treatment, angiotensin II infusion and sodium restriction (Malee & Mellon, 1991; Shibata *et al*, 1991; Imai *et al*, 1992b).

Similar isoforms of P450c11 have since been identified in mouse adrenal cortex (Domalik *et al* 1991), and mRNA encoding the aldosterone-synthetic isoform was identified in hyperplastic adrenocortical tissue from human patients with primary aldosteronism (Kawamoto *et al*, 1990; Ogishima *et al*, 1991) and in cultured human zg cells (Curnow *et al*, 1991). Thus the presence of two distinct P450c11 enzymes provides a mechanism for the selective synthesis of aldosterone in rat, mouse and human adrenal cortex. However, the situation in the bovine adrenal gland is less well understood. Although cDNAs encoding two different cytochromes P450c11 have been cloned and sequenced in this species (Kirita *et al*, 1988), both isoforms are present throughout the adrenal cortex, and show identical enzymatic activities (ie. synthesise cortisol from 11-deoxycortisol and aldosterone from corticosterone) when expressed in COS-7 cells (Morohashi *et al*, 1990). It is possible that in the inner bovine adrenocortical zones, the

local mitochondrial environment does not support the aldosterone synthase activity of P450c11, perhaps influenced by the steroid gradient across the cortex, although this hypothesis has not been tested to date.

1.3 STIMULATION OF STEROIDOGENESIS

1.3.1 The hypothalamic-pituitary-adrenal axis

The major hormonal stimulus to glucocorticoid secretion from the adrenal cortex is adrenocorticotropin (ACTH), a 39-amino acid peptide derived from proopiomelanocortin (POMC). The most potent adrenocortical agonist known, ACTH has been reported to stimulate glucocorticoid secretion from isolated adrenocortical cells at concentrations as low as 10^{-16}M (Seelig & Sayers, 1973). In addition to its steroidogenic effect on both cortisol and aldosterone secretion, ACTH also increases blood flow through the adrenal by inducing vasodilatation of the adrenocortical blood vessels (reviewed in Vinson *et al*, 1985). Long term ACTH treatment induces both hypertrophy and hyperplasia of adrenocortical cells, and is associated with a marked depletion of cellular lipid and reduction in adrenocortical cholesterol content.

Synthesised within, and released from, the basophilic corticotrophs of the anterior pituitary, ACTH secretion is regulated almost exclusively by corticotropin- releasing hormone (CRH), a 41-amino acid peptide whose structure was not identified until 1981 (Spiess *et al*, 1981). Within the CNS, CRH is found mainly within the parvocellular region of the hypothalamic paraventricular nucleus (PVN). These neurons form dense projections to the median eminance, where neurally-released CRH gains access to the hypothalamohypophysial portal system and thus to the pituitary. In addition, immunoreactive CRH has been detected in many other brain areas and peripheral tissues including cerebral cortex, limbic system, brainstem nuclei, lung, adrenal medulla, gut and testes, where the peptide is thought to act as a neurotransmitter (reviwed in Owens & Nemeroff, 1991).

CRH stimulates the release of pituitary ACTH (and other POMC-derived peptides such as β-endorphin) both *in vitro* (Vale *et al*, 1983) and *in vivo* (Donald *et al*, 1983). In addition, a trophic action of CRH on pituitary corticotrophs has been reported (Westlund *et al*, 1985; McNicol *et al*, 1988). It is generally agreed that these actions are mediated by a single class of CRH receptor positively coupled to adenylyl cyclase (Ur & Grossman, 1992), although stimulation of phosphatidylinositol hydrolysis has been

reported to occur in response to CRH in rat hypothalamic slices (Owens & Nemeroff, 1991).

(i) Regulation of CRH release

The secretion of CRH is influenced by a complex interaction of nervous and humoral factors. Immunocytochemical studies (reviewed by Ownes & Nemeroff, 1991) have provided evidence for synaptic connections between CRH-containing neurones in the PVN and noradrenergic, adrenergic, cholinergic, serotonergic, dopaminergic and GABAergic fibres arising from higher brain centres, as well as possible autoregulatory interactions between CRH neurones themselves. The effects of acetylcholine, noradrenaline and serotonin appear to be stimulatory (Tsagarakis *et al*, 1988; Calogero *et al*, 1989), whereas those of GABA are inhibitory (Ur & Grossman, 1992). The secretion of CRH is also inhibited by other components of the HPA axis. Thus, both glucocorticoids and ACTH, as well as CRH itself, inhibit the release of hypothalamic CRH, representing long, short and ultrashort feedback loops respectively (Calogero *et al*, 1988).

(ii) Regulation of ACTH release

Although CRH is the major stimulus to ACTH secretion in most species, other neuropeptides possess ACTH-releasing activity and may be important in the regulation of the HPA axis either alone, or in concert with CRH. For example, arginine vasopressin (AVP) weakly stimulates ACTH release by itself, but exerts a marked synergism with CRH in this respect (Gillies *et al*, 1982; Fischman & Moldow, 1984). Similar potentiation of the effects of CRH have also been observed with oxytocin, angiotensin II, neuropeptide Y and the intestinal peptide PHI-27 (Gibbs *et al*, 1984; Schoenenberg *et al*, 1987; Koenig, 1990; Tilders *et al*, 1984). It should be noted that in ovine species, AVP rather than CRH appears to be the major stimulus to ACTH secretion (Familari *et al*, 1989).

(iii) Regulation of CRH and ACTH release by glucocorticoids

Circulating glucocorticoids exert a negative feedback influence at various sites within the HPA axis. Glucocorticoid receptors exist on CRH-containing neurons in the hypothalamic PVN (Uht *et al*, 1988), and stress-induced increases in hypophysial portal vessel CRH concentration can be blocked by dexamethasone treatment or increased

plasma corticosterone concentration (Plotsky & Vale, 1984; Plotskey *et al*, 1986). In addition, evidence exists to suggest that glucocorticoid receptors in higher brain centres such as the hippocampus may be involved in the negative feedback of glucocorticoids on CRH release (Sapolsky *et al*, 1989).

Glucocorticoid feedback inhibition of ACTH secretion also occurs at the level of the pituitary, and comprises two phases: a rapid onset inhibition (occuring within hours) of CRH-stimulated, but not basal, ACTH secretion, and a slower component (days) during which both basal and CRH-stimulated ACTH secretion are impaired (see Ur & Grossman, 1992). This long-term effect is associated with a decrease in pituitary CRH receptor number (Hauger *et al*, 1987). The interactions between the various components of the HPA axis are shown diagramatically in Figure 1.6.

1.3.2 The intra-adrenal CRH-ACTH axis

Recent observations have suggested the presence of both CRH and ACTH within the adrenal gland itself, confined mainly to the adrenal medulla, raising the possibility that the adrenal gland may possess an active CRH-ACTH system which may be involved in the regulation of steroidogenesis. CRH-like immunoreactivity has been observed in human phaeochromocytoma tissue and bovine adrenal medulla (Suda *et al*, 1986; Minamino *et al*, 1988), and the presence of ACTH within the rat adrenal gland has been suggested by the stimulatory effects of exogenous CRH on steroidogenesis (Andreis *et al*, 1991). Indeed, ACTH immunoreactivity was reported in the human adrenal medulla (Suda *et al*, 1986), and POMC gene expression reported in the bovine adrenal medulla (Throne *et al*, 1991). That secretion of these trophic hormones may occur under physiological conditions is suggested by the increase in adrenal vein POMC and ACTH concentrations reported to occur on stimulation of the splanchnic nerve in the bovine species (Jones & Edwards, 1990).

1.3.3 The renin-angiotensin system

The renin-angiotensin system (RAS) was 'discovered' almost one hundred years ago following the observation that a saline extract of the kidney was capable of producing a rise in blood pressure. This hypertensive renal extract was subsequently shown to contain the enzyme renin, and later work demonstrated that renin catalysed the conversion of a plasma protein to yield an active pressor substance. In 1940, the pressor substance was identified independently by two groups who named it angiotonin and hypertensin

FIGURE 1.6

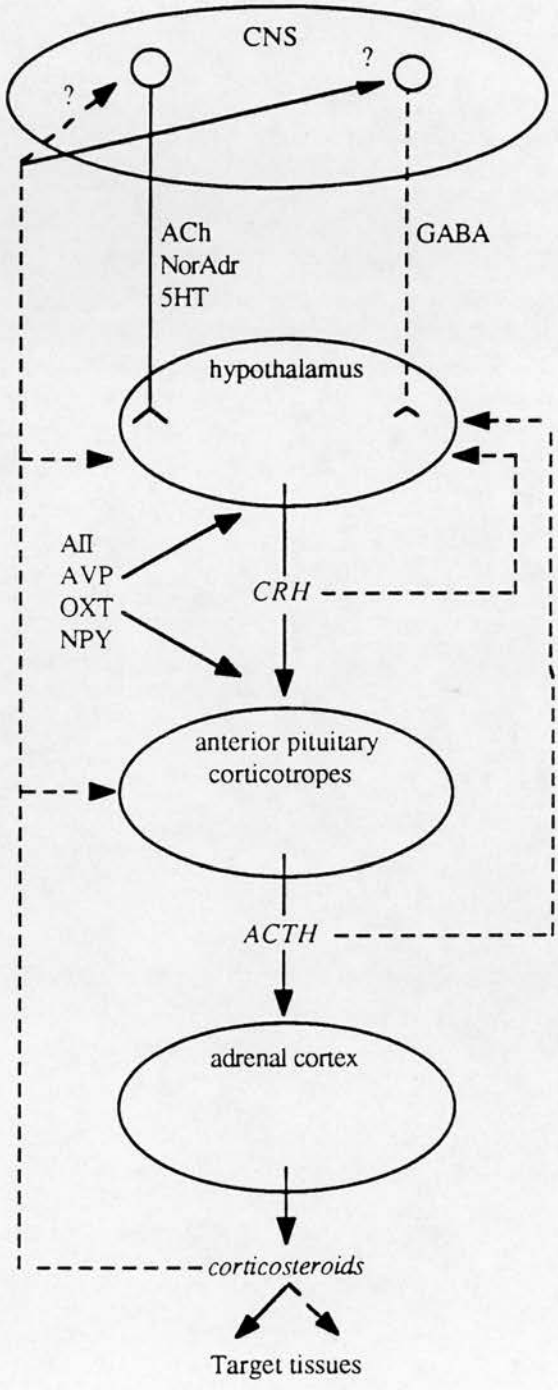

FIGURE 1.6 : The hypothalamic-pituitary-adrenal axis. Stimulatory and inhibitory pathways are indicated by solid and dashed lines respectively. Question marks indicate putative interactions. Abbreviations: CNS, central nervous system; ACh, acetylcholine; NorAdr, noradrenaline; 5HT, serotonin; GABA, γ-amino butyric acid; ANP, atrial natriuretic peptide; SubP, substance P; AII, angiotensin II; AVP, arginine vasopressin; OXT, oxytocin; NPY, neuropeptide Y; CRH, corticotropin releasing hormone; ACTH, corticotropin.

respectively, and in 1958 the internationally agreed nomenclature of angiotensin was adopted.

Renin is a 42K proteolytic enzyme secreted principally by the juxtaglomerular cells of the kidney, which store the enzyme in intracellular granules. Renin acts on a 57K plasma $\alpha 2$ globulin, angiotensinogen, cleaving it at the leu_{10}-leu_{11} bond to produce the biologically inactive decapeptide angiotensin I (AI) (see Figure 1.10, section 1.6). AI is further metabolised by angiotensin converting enzyme (ACE) to produce the active octapeptide AII. ACE, located primarily in the pulmonary vascular bed, is a relatively non-specific peptidase, and also metabolises peptide inflammatory mediators such as bradykinin. AII has a relatively short plasma half life (ca. 60 sec), being rapidly broken down by angiotensinase peptidases to produce inactive peptide fragments.

The physiological actions of AII are many and varied, and in concert act to maintain blood pressure and volume. Next to endothelin, AII is the most potent pressor substance known (ca. 40 times more potent than noradrenaline (mol/mol)), and produces intense vasoconstriction in most vascular beds, especially those of the skin, kidney and intestines. AII potently stimulates adrenocortical steroidogenesis, and stimulation of aldosterone secretion can be observed at sub-constrictor doses (picomolar). AII also directly stimulates sodium reabsorption in the renal proximal tubule, and enhances sympathetic nerve transmission by stimulating noradrenaline release from peripheral nerve endings, stimulating tyrosine hydroxylase activity and inhibiting noradrenaline uptake into nerves. Central effects of AII contribute to this pressor activity: AII reduces the vagal inhibitory tone of the heart via stimulation of the area postrema, and promotes thirst and drinking behaviour via AII receptors located in the subfornical organ and paraventricular nuclei.

The RAS is activated in conditions of sodium depletion, hypotension and congestive heart failure. Thus, sodium depletion stimulates release of renin and subsequent formation of AII, and the resulting increase in aldosterone secretion leads to enhanced renal sodium resorption. The adrenal sensitivity to AII is also increased through increases in AII receptor number and affinity (Aguilera *et al,* 1978). A fall in blood pressure, due either to excess vasodilation or fluid loss also results in increased release of renin. This effect is mediated by sympathetic stimulation of the renal juxtaglomerular cells following baroreceptor inactivation, and can be mimicked by injection of β_1-adrenergic agonists. Increased plasma renin activity due to enhanced sympathetic nerve activity can also be measured following a change in posture from the supine to erect position. The role of the RAS in the control of blood pressure and electrolyte homeostasis is reviewed in Hall *et al* (1990).

1.3.4 The intra-adrenal renin-angiotensin system

In 1969, a renin-like enzyme was identified in the rabbit adrenal gland (Ryan, 1969). Since this observation, renin has been found in many tissues including ovary, testes, uterus, pituitary, heart, vascular smooth muscle, brain and adrenal (Field *et al,* 1984; Dzau *et al,* 1987; Suzuki *et al.* 1988; Deschepper *et al,* 1986). Since the adrenal cortex is a major target tissue for AII, much study has been made of the significance of intra-adrenal renin. Renin has been identified in the adrenal cortex of rat, murine, canine, bovine and human species (Ganten *et al,* 1974, 1976; Hayduk *et al,* 1970; Ryan, 1972; Naruse *et al,* 1983), and the demonstration of renin mRNA in rat mouse and human adrenal cortex suggests that adrenal renin is synthesised locally and not simply taken up from the circulation (Deschepper *et al,* 1986; Field *et al,* 1984; Shionoiri *et al,* 1992). Furthermore, in rat adrenal cortex there exists at least the potential for an active intra-adrenal RAS since in this tissue, all the components of the peripheral RAS have been identified (renin, prorenin, angiotensinogen, angiotensins I and II, angiotensin converting enzyme) (Reviewed in Mulrow *et al,* 1988).

The physiological significance of adrenal renin has been a subject of much debate in recent years. Localised mainly in the outer zg of the rat adrenal (Brecher *et al,* 1989), increasing evidence suggests that adrenal renin release may be regulated by the same factors which stimulate release of renal renin. Thus, adrenal renin was increased in sodium depleted or potassium loaded rats (Doi *et al,* 1984). In cultured bovine zg cells, cellular renin activity and release of renin were stimulated by catecholamines and dibutyryl cAMP (Gupta *et al,* 1992). This is particularly interesting in view of the demonstration of a sympathetic innervation of the adrenal cortex (Gilchrist *et al,* 1993; Charlton *et al,* 1992), and the known involvement of sympathetic tone in the release of renal renin (Osborn & Johns, 1989).

A number of observations suggest that locally synthesised renin may regulate aldosterone secretion. In cultured rat zg cells, ACTH and potassium stimulated both aldosterone secretion and renin activity (Yamaguchi *et al,* 1990). This increase in renin activity was associated with elevated levels of renin mRNA (Wang *et al,* 1992). Furthermore, lisinopril, an ACE inhibitor, reduced the aldosterone secretory response to ACTH and potassium without affecting renin activity (Yamaguchi *et al,* 1990). Similar inhibitory effects of ACE inhibitors on ACTH-stimulated aldosterone secretion have been reported in humans *in vivo* (Ramirez *et al,* 1988).

Less information is available regarding the renin content of zfr cells. Renin is present in both the zg and inner zfr of the mouse adrenal cortex, and in some species of mouse is mainly present in the zfr (Naruse *et al,* 1984). By contrast, rat adrenal renin is confined to the zg (Brecher *et al,* 1989). Significantly, TGR (mRen-2)27 rats (rats transfected with the Ren-2 mouse renin gene which causes severe hypertension (Mullins *et al,* 1990)) differ from normal rats in that their adrenal renin is elevated and present in both the zg and zfr (Yamaguchi *et al,* 1992). While bovine zg cells in culture clearly synthesise renin (Gupta *et al,* 1992), the renin content of the bovine inner zone is unknown. However, it seems likely, given the centripetal nature of the adrenocortical blood supply, that AII synthesised locally within the zg would be accessible to the inner zfr, even if zfr cells do not themselves possess an active local RAS.

1.3.5 Other factors

Until relatively recently it was widely assumed that aldosterone secretion from the zg was controlled exclusively by the renin angiotensin system, while ACTH was considered to be the sole stimulus to cortisol secretion from the inner adrenocortical zones. However, it is now becoming clear that the regulation of steroidogenesis is multifactorial, and a variety of different hormones and neurotransmitters have been reported to stimulate (or inhibit) adrenocortical steroidogenesis (reviewed in Tait et al, 1980a; Bird et al, 1990b). Table 1.2 summarises reported agonist effects on steroidogenesis in adrenocortical preparations. It is emphasised that the data in Table 1.2 represents a compilation of results from *in vitro* and *in vivo* studies in several species, and is intended only to illustrate the wide variety of agonists which are capable of stimulating steroidogenesis. Several important species differences exist. For example, serotonin stimulates aldosterone secretion from rat, but not bovine zg cells, and angiotensin II is a potent stimulus for bovine, but not rat zfr cells. A more detailed consideration of agonist effects on steroid secretion and second messenger formation in bovine adrenocortical cells is given in Table 1.3.

With the exception of potassium, which stimulates aldosterone secretion through a receptor-independent mechanism, the agonists detailed in Table 1.3 stimulate steroidogenesis by activating either the adenylate cyclase/cAMP signalling system or the inositol phosphate/calcium signalling system. Rather than consider each agonist in turn, the general mechanisms by which activation of adenylate cyclase and stimulation of phosphatidylinositol turnover lead to increased steroidogenesis will be considered. More detailed descriptions of the mechanisms by which acetylcholine and angiotensin II stimulate steroidogenesis in adrenocortical cells will follow in sections 1.5 and 1.6.

TABLE 1.2

Agonist	*Steroidogenesis* mineral.	Glucocort.	*Comments*	*Reference*
ACTH	+	+	In all species studied	1,2
AII	+	+	Not steroidogenic in rat/ovine zfr cells. Via AT_1 receptor in bovine zg	1-3
Acetylcholine	+	+	Via PLC in bovine zg and zfr cells	2,4-6
Adrenaline	+	+	{ Not steroidogenic in rat zfr cells	7-9,55
Noradrenaline	+	+	{Via ß1 receptor in bovine zfr cells	
Serotonin	+	+	$5HT_4$ receptor-mediated effect on cortisol in human tissue and corticosterone in frog tissue	10-12, 19,20
Dopamine	-	?	Inhibits agonist-stimulated aldosterone secretion in rat and bovine zg cells	13-15
AVP	+	+	Stimulates aldosterone in rat and bovine zg, cortisol in bovine zfr. AVT stimulates corticosterone in frog	16-18
Adenosine	?	-	Inhibits basal and ACTH-stimulated corticosteroid in rat via A_1 receptor	49
ATP	+	+	Stimulates cAMP and Ca^{2+} in bovine zfr cells	48,50,51
ANF	-	-	Potently inhibits AII-stimulated aldosterone in rat and bovine zg. Inhibits cortisol in bovine zfr	21-24
BNP	-	-	In cultured bovine and human adrenocotrical cells	24,25
Substance P	- (rat)	+	Ca2+/calmodulin dependent in BAC. Inhibits basal and ACTH-stimulated corticosterone in rat	26-28

/cont.

TABLE 1.2 (continued)

Agonist	*Steroidogenesis* *mineral.*	*Glucocort.*	*Comments*	*Reference*
Somatostatin	-	?	Tonic inhibitory effect on aldosterone in rat zg both *in vitro* and *in vivo*	29-32
α-MSH	+	+	Stimulates both rat zg and zfr cells. Linked to cAMP in bovine zg cells	33-36
VIP	+	+	Possibly via release of medullary catecholamines in intact rat gland	37-39
Endothelins	+	+	In rat, bovine and human zg and zfr cell suspensions	40-45
NKA/B	?	+	In cultured BAC	27
NPY	-	?	Inhibits basal and ACTH-stimulated aldosterone/18-OH corticosterone in isolated rat zg cells.	28,46
ß-Endorphin	?	?	Potentiated ACTH- and α-MSH-stimulated corticosterone in rat zfr cells	35,47
ß-Lipotropin	+	?	In rat and bovine zg cells	36
Interleukins	?	+	Prostaglandin-dependent stimulation of corticosterone in rat adrenocortical cells. Not steroidogenic in bovine zfr	52,53
PTH	+	?	Stimulates aldosterone in bovine zg cells. Also synergistic with AII	54

TABLE 1.2 : Reported agonist-stimulated steroidogenic responses of adrenocortical preparations. Original references are given overleaf. Abbreviations: ACTH, adrenocorticotropin; AII, angiotensin II; AVP, arginine vasipressin; AVT, arginine vasotocin; ATP, adenosine trisphosphate; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; α-MSH, α-melanocyte-stimulating hormone; VIP, vasoactive intestinal peptide; NKA/B, neurokinin A/B; NPY, neuropeptide Y; PTH, parathyroid hormone; PLC, phospholipase C; BAC, unpurified bovine adrenocortical cells.

TABLE 1.2 (continued)

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47. Szalay, 1993
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49. Feuilloley *et al*, 1992
50. Kawamura *et al*, 1991
51. Matsui, 1991
52. Tominaga *et al*, 1991
53. Hanley *et al*, 1992
54. Isales *et al*, 1991
55. Kawamura *et al*, 1984

TABLE 1.3

	zona glomerulosa			*zona fasciculata/reticularis*		
agonist	*steroid*	*PtdIns*	*cAMP*	*steroid*	*PtdIns*	*cAMP*
ACTH	+ (1,2)	0 (1,2)	+ (1,2)	+ (1)	?	+ (1)
AII	+ (1,2)	+ (1,2)	0 (3)	+ (4)	+ (4,5)	0 (6,7)
adr	+ (8)	?	?	+ (9,10)	0 (7)	+ (9,10)
serotonin	0 (11)	?	?	0 (11)	?	?
dopamine	- (12)	?	?	?	?	?
α–MSH	+ (13)	0 (13)	+ (13)	?	?	?
ACh	+ (14)	+ (14)	0 (14)	+ (15,16)	+ (16,17)	0 (15,16)
AVP	?	?	?	+ (18)	+ (18)	0 (18)
ANP	- (2,19)	?	- (19)	?	?	?
ATP	+ (20)	?	?	+ (21,22)	+ (21,22)	+/0 (21,22)
potassium	+ (23)	+ (23)	+ (23)	?	?	?

+, stimulatory effect; -, inhibitory effect; 0, no effect; ?, no data available

TABLE 1.3 : Acute steroid and second messenger responses in purified bovine adrenocortical cell preparations. Original references are given overleaf. Abbreviations: ACTH, adrenocorticotropin; ANP, atrial natriuretic peptide; AII, angiotensin II; adr, adrenaline; ACh, acetylcholine; AVP, arginine vasopressin; ATP, adenosine trisphosphate; α-MSH, α-melanocyte stimulating hormone.

TABLE 1.3 (continued)

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19. McFarland *et al*, 1991
20. G. Dell, personal communication
21. Nitsu *et al*, 1992
22. Hoey *et al*, 1994
23. Kojima *et al*, 1985e

1.3.6 Adenylate cyclase

Numerous hormones and neurotransmitters exert their cellular effects by increasing the levels of the intracellular second messenger cAMP in their target cells. The concentration of cAMP is regulated by two separate enzymes: adenylate cyclase, which catalyses the formation of cAMP from ATP, and cAMP-specific phosphodiesterase, which breaks down cAMP to form 5'AMP. Increased levels of cAMP lead to the activation of cAMP-dependent protein kinases, which in turn increase protein phosphorylation, resulting in the measured cellular response.

Hormonally-regulated adenylate cyclase, first described by Sutherland and co-workers (Robison *et al,* 1967), is a ubiquitous multicomponent system comprised of five functional units (Figure 1.7). The transmembrane catalytic unit which hydrolyses ATP is linked to agonist receptors which either stimulate the enzyme via a stimulatory G protein (G_s) or inhibit it via an inhibitory G protein (G_i). G_s and G_i are each composed of three subunits α, β and γ. The β and γ subunits of both G_s and G_i are highly homologous (Hildebrandt *et al,* 1984) and exist as tightly associated complexes. The α subunits, though also highly homologous, are structurally different, and the identity of the various α subunits are used to define a given G protein heterotrimer. At present, 12 different α subunits of G_s or G_i have been characterised, of which two are known to stimulate adenylate cyclase ($\alpha_{s(s)}$ and α_{olf}), and two to inhibit the enzyme (α_{i3} and α_z) (Hepler & Gilman, 1992). The α subunits of the G_s family are targets for ADP-ribosylation by cholera toxin, while those of the G_i family can be ADP-ribosylated by pertussis toxin (although α_{t1} and α_{t2}, which stimulate cGMP-specific phosphodiesterase, appear to be susceptible to both toxins (Hepler & Gilman, 1992)). The mode of coupling of adenylate cyclase to stimulatory hormone receptors is reviewed in detail in Levitzki (1986).

Agents known to stimulate adrenocortical steroidogenesis via adenylate cyclase include ACTH and β-adrenergic agonists (Table 1.3). However, the majority of information regarding the mechanism of cAMP-induced steroidogenesis in adrenocortical cells has come from the study of ACTH.

1.3.7 Mechanism of action of ACTH in adrenocortical cells

Numerous studies have established that ACTH increases the concentration of cAMP in adrenocortical cells, through activation of adenylate cyclase (Tait *et al,* 1980a). The principal locus of the steroidogenic action of ACTH is the conversion of cholesterol to pregnenolone (Koritz & Kumar, 1970), the major rate-limiting step in the steroidogenic

FIGURE 1.7

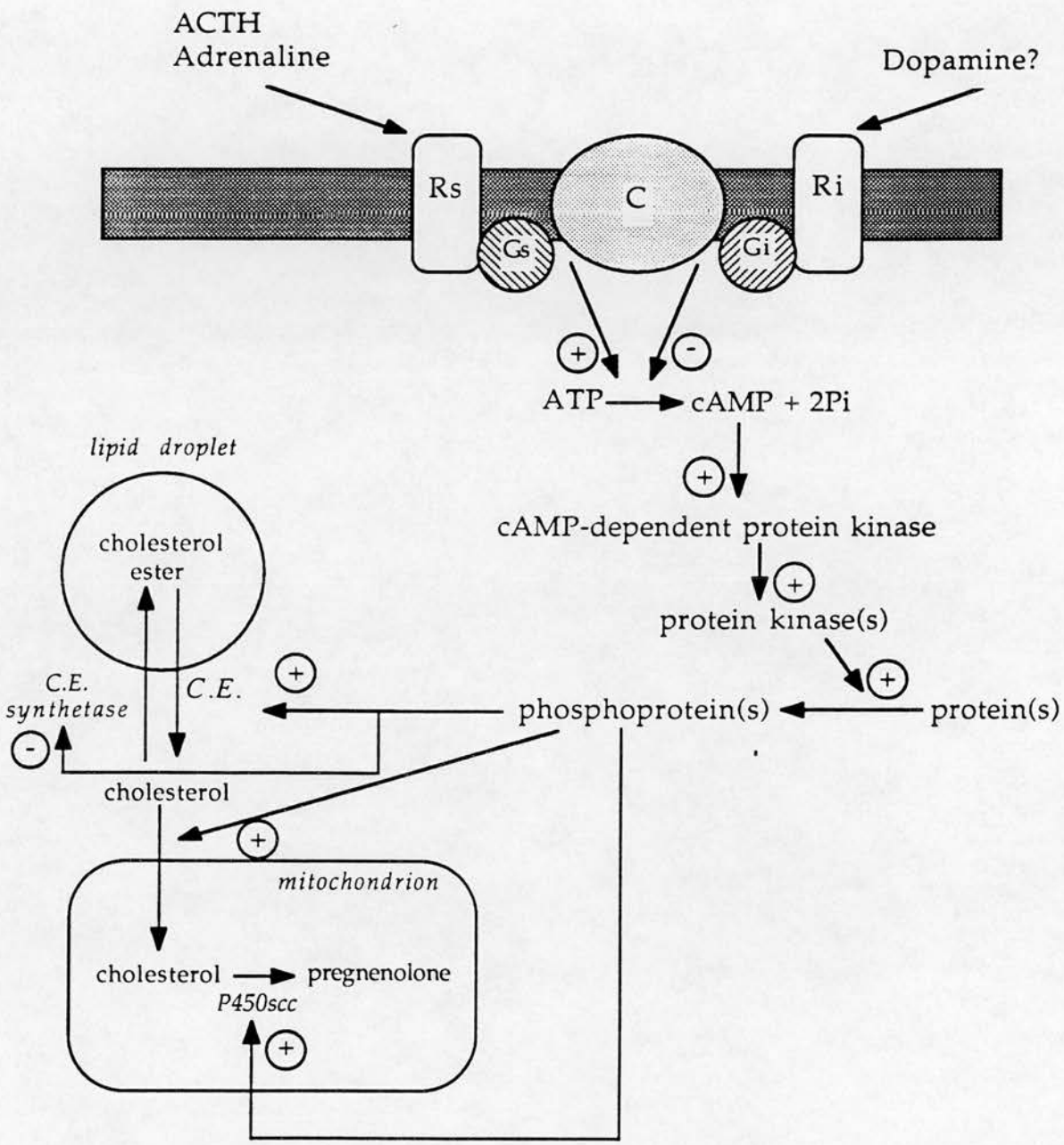

FIGURE 1.7 : Structure of hormonally regulated adenylate cyclase. Steroidogenic agonists such as ACTH interact with stimulatory receptors (R_S) which activate the enzyme catalytic unit (C) via a pertussis toxin-insensitive G protein (Gs). The enzyme can also be inhibited by agents which interact with receptors linked to inhibitory G proteins (Gi). cAMP thus formed on activation of stimulatory receptors stimulates cAMP-dependent protein kinases, eventually leading to activation of steroidogenesis. Steroidogenic enzymes known to be phosphorylated in response to adenylate cyclase agonists include cholesterol esterase (*C.E.*), cholesterol ester synthetase (*C.E. synthetase*) and P450 side-chain cleavage (*P450scc*).

pathway (Stone & Hechter, 1955). The effects of increased intracellular cAMP levels in response to ACTH are reviewed in Tait *et al* (1980a) and Hornsby (1988), and include phosphorylation of cholesterol esterase and cholesterol ester synthetase, leading to the respective activation and inactivation of these enzymes and a consequent increase in the availability of free cholesterol from cholesterol ester, enhancement of cholesterol transport into mitochondria, and facilitation of cholesterol binding to P450scc. In addition, ACTH has been reported to enhance the release of newly-formed pregnenolone from P450scc. These effects of ACTH are believed to be mediated by a cAMP-dependent protein kinase, since protein phosphorylation is an early event in the steroidogenic action of ACTH (Koroscil & Gallant, 1980; Bristow *et al,* 1981; Mikami & Strott, 1986). Indeed, inhibitors of protein kinase A dose-dependently inhibit the aldosterone-secretory response of rat zg cells (Kurscheid-Reich *et al,* 1992). However, the exact substrates for protein kinase-mediated phosphorylation have not been identified, although several phosphoproteins have been proposed as candidates. For example, a 28K phosphoprotein was identified in rat adrenal cortex mitochondria which was produced with a time course and concentration-dependence similar to that of ACTH-stimulated aldosterone secretion (Epstein *et al,* 1989). ACTH-stimulated phosphorylation of a cytoplasmic protein (APS150) has also been reported (Podesta *et al,* 1979).

These acute actions of ACTH are confined to the P450scc-mediated synthesis of pregnenolone. ACTH does however exert long-term chronic effects directly at the level of the steroidogenic enzymes. The production of P450scc mRNA in bovine adrenocortical cells is abolished in the absence of ACTH (DuBios *et al,* 1981). Thus, the presence of ACTH appears to be necessary for P450scc synthesis. Indeed, both ACTH and cAMP stimulate the accumulation of P450scc mRNA in bovine adrenocortical cells (Simonian *et al,* 1979b). ACTH has also been reported to stimulate the synthesis of other steroidogenic enzymes including P450c21, P450c11, P450c17 and adrenodoxin in bovine adrenocortical cells, and increase mRNA levels for P450scc and P450c17 in human foetal adrenal cells (reviewed in Miller, 1988).

1.3.8 Adrenocortical ACTH receptors

The identification and characterisation of adrenocortical ACTH receptors has been hampered in the past by the relatively low biological activity of radiolabelled ACTH. More recently, the availability of ^{125}I-labelled ACTH analogues has allowed the identification of ACTH binding sites in adrenocortical preparations from several species including rat, sheep, cattle and human (Penhoat et al, 1991 and references therein). The binding affinity of ACTH was found to be similar in these preparations (K_D=0.2 nM). A

second low affinity/high capacity binding site (K_D=2-10nM) has been reported in rat (Gallo-Payet & Escher, 1985) and bovine (Penhoat et al, 1989) adrenal cortex, although this remains controversial (Penhoat et al, 1991).

A second approach to the characterisation of ACTH receptors has been covalent cross-linking of ^{125}I-ACTH with disuccinimidyl suberate to adrenocortical preparations. Such studies have shown the ACTH receptor of bovine zfr cells and human adrenal cortex membranes to be a membrane-associated protein of Mr=43kD (Penhoat et al, 1993). In bovine adrenal cortex membrane fractions, affinity labelling experiments identified an ACTH-binding protein of Mr=40kD (Mizuno et al, 1989). Although these studies provide a first step for the purification and cloning of the ACTH receptor, this has proved to be extremely difficult, since solubilisation of the receptor destroys its ligand binding capability.

The ACTH receptor was cloned by Mountjoy et al (1992) who performed polymerase chain reaction using cDNA from a human melanoma rich in MSH receptors. Two clones were identified which encoded functional MSH and ACTH receptors respectively. The ACTH receptor, although containing the seven predicted transmembrane domains characteristic of the G protein-coupled receptor superfamily, represents the smallest such receptor thus far cloned, at 297 amino acids. This predicted amino acid sequence corresponds to a protein of around 35kD, suggesting that the biochemical studies discussed above identified the glycocylated form of the receptor. Functional coupling to adenylate cyclase was demonstrated in Cloudman S91 cells transfected with human ACTH receptor DNA, which showed a ten-fold elevation of cAMP in response to physiological concentrations of ACTH (1nM) (Mountjoy et al, 1992). Expression of ACTH receptor mRNA in rhesus monkey adrenal gland was concentrated in the zf, but also present in the zr and inner regions of the zg. No receptor mRNA was detected in the capsule or medulla.

1.3.9 Phosphatidylinositol metabolism

The understanding of the cellular mechanisms involved in the steroidogenic action of ACTH and other agonists which stimulate adenylate cyclase advanced markedly during the 1970s and 1980s, and the relationships between hormone stimulated cAMP generation and activation of steroidogenesis became clear. However, it was observed that some agonists stimulated steroidogenesis without increasing cAMP levels, but with an accompanying increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$). At this time, the involvement of phosphoinositide turnover in the cellular action of agonists in

other systems was established, and it was accepted that at least two independent mechanisms, adenylate cyclase and phosphatidylinositol turnover, were involved in the regulation of adrenocortical steroidogenesis.

Hormone-stimulated metabolism of membrane phosphoinositides was first described by Hokin and Hokin (1953), who demonstrated increased incorporation of ^{32}P into phospholipids in response to ACh in the pancreas. The link between this metabolism of phospholipids and agonists which stimulate increases in $[Ca^{2+}]_i$ was made by Michell (1975), and the importance of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) in regulating intracellular Ca^{2+} was established (Irvine & Berridge, 1984).

The major pathways of phosphoinositide metabolism are shown in Figure 1.8. Phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$), a minor membrane phospholipid accounting for less than 1% of total cellular inositol (Michell *et al*, 1970), is produced by a two-stage phosphorylation of phosphatidylinositol (PtdIns), and dephosphorylated by specific phosphomonoesterases, creating a dynamic equilibrium between PtdIns, phosphatidylinositol 4 phosphate (PtdIns4P) and $PtdIns(4,5)P_2$. $PtsIns(4,5)P_2$ is rapidly degraded by the action of hormone-sensitive phospholipase C during agonist activation of receptors to form the second messengers $Ins(1,4,5)P_3$ and diacylglycerol (DAG). $Ins(1,4,5)P_3$ is rapidly inactivated by sequential dephosphorylation by intracellular phosphatases to form free inositol via $Ins(1,4)P_2$ and Ins(4)P. Alternatively, $Ins(1,4,5)P_3$ can be further phosphorylated to inositol tetrakisphosphate ($Ins(1,3,4,5)P_4$) before dephosphorylation via $Ins(1,3,4)P_3$, $Ins(1,4)P_2$ and Ins(3)P to form free inositol. The final dephosphorylation of the inositol monophophosphates to free inositol can be inhibited by lithium, the inclusion of which in experimental media allows the accumulation of inositol phosphates for assay. DAG is converted via phosphatidic acid (PA) to cytidine diphosphodiacylglycerol (CDP-DAG) which combines with free inositol to reform PtdIns. Thus, although stimulation of phospholipase C results in increased breakdown of $PtdIns(4,5)P_2$, the net effect is to increase phosphoinositide turnover, rather than to decrease phosphoinositide mass.

The cleavage of $PtdIns(4,5)P_2$ by PLC results in the formation of two independent intracellular second messengers, $Ins(1,4,5)P_3$ and DAG, which act to mobilise $[Ca^{2+}]_i$ and stimulate protein kinase C (PKC) respectively (reviewed in Berridge, 1987; Berridge & Irvine, 1989).

FIGURE 1.8

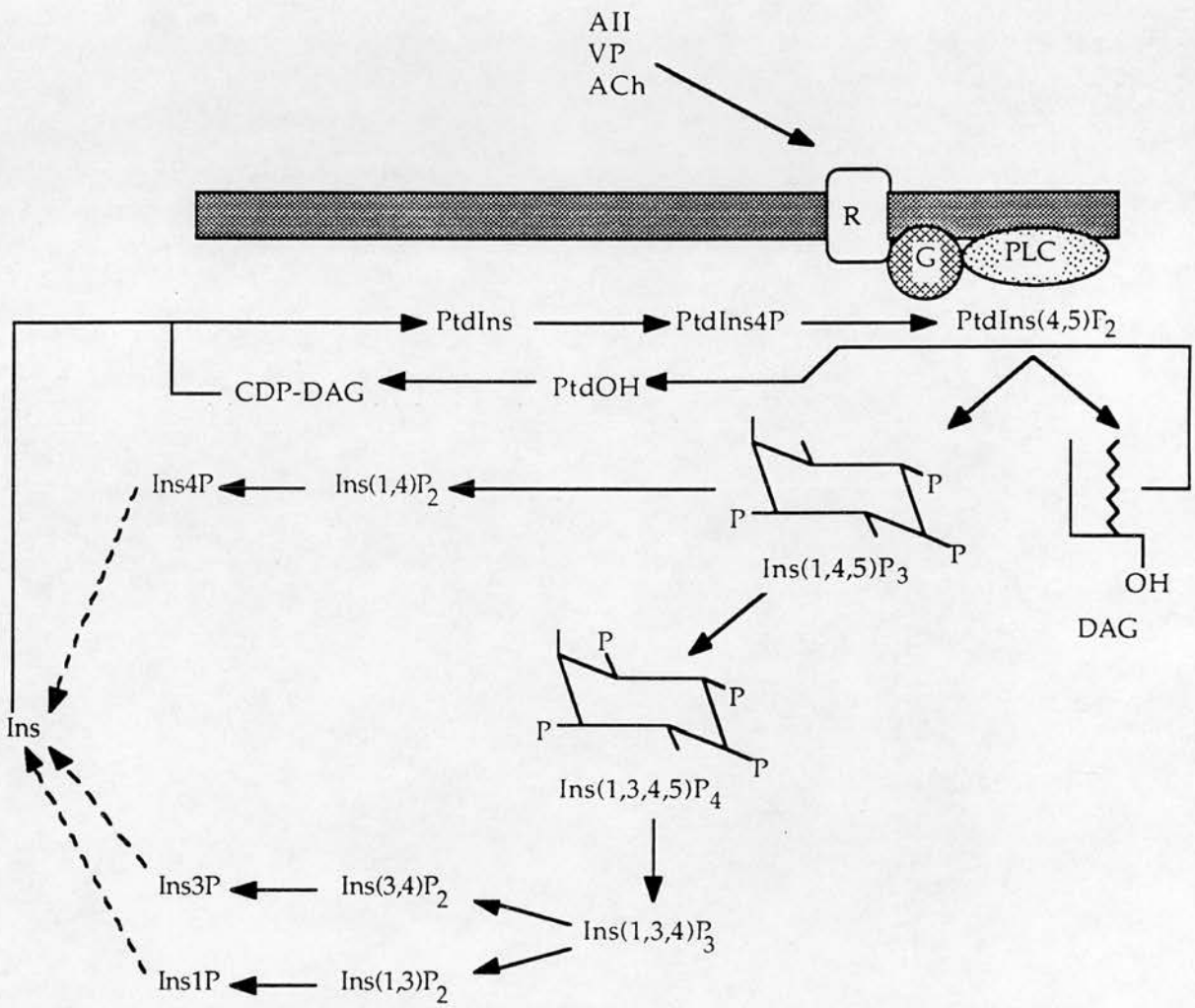

FIGURE 1.8 : Phosphoinositide metabolism in adrenocortical cells. Phospholipase C (PLC), activated in response to receptor (R) occupancy via a stimulatory G protein (G), hydrolyses phosphatidylinositol(4,5) bisphosphate (PtdIns(4,5)P_2) to produce the second messengers inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). Ins(1,4,5)P_3 is recycled back to free inositol (Ins) by sequential phosphorylation/dephosphorylation, the final steps of which are inhibited by lithium (dotted lines). DAG is recycled through conversion to phosphatidic acid (PtdOH) and cytidine diphosphodiacylglycerol (CDP-DAG), which combines with free inositol to reform phosphatidylinositol (PtdIns). The aqueous phosphoinositols and free inositol are cytosolic; all other species shown here are associated with the plasma membrane.

1.3.10 Ins(1,4,5)P_3 and Ca^{2+} mobilisation

Calcium mobilising agonists frequently stimulate a biphasic Ca^{2+} response in target cells. The initial response, a transient mobilisation of Ca^{2+} from intracellular stores, is followed by a more sustained influx of extracellular Ca^{2+}. Inositol phosphates are thought to play a role in both phases of Ca^{2+} mobilisation.

The link between Ins(1,4,5)P_3 and Ca^{2+} mobilisation was established in 1983. Addition of Ins(1,4,5)P_3 to permeablised pancreatic cells resulted in the release of Ca^{2+} from endoplasmic reticulum (Sterb *et al*, 1983). Many subsequent cell fractionation studies have confirmed that Ins(1,4,5)P_3 releases Ca^{2+} from a membrane fraction derived exclusively from the endoplasmic reticulum (Rana & Hokin, 1990) The size of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool appears to vary markedly between cell types from around 20% of endoplasmic reticular Ca^{2+} in adipocyte membrane fractions (Delfert *et al*, 1986) to around 90% in permeablised insulinoma (RINm5F) cells (Prentki *et al*, 1985).

Ins(1,4,5)P_3 mobilises Ca^{2+} by binding to specific receptors which promote the opening of endoplasmic reticulum Ca^{2+} channels. The Ins(1,4,5)P_3 receptor undergoes a conformational change on binding of Ins(1,4,5)P_3 which is thought to be involved in the coupling of the receptor to Ca^{2+} channel opening (Berridge, 1993). It has been estimated that the binding of one Ins(1,4,5)P_3 molecule promotes the release of around 20 Ca^{2+} ions (Joseph *et al*, 1984).

The sustained rise in $[Ca^{2+}]_i$ observed on agonist stimulation occurs via influx of extracellular Ca^{2+}. Although Ins(1,4,5)P_3 does not itself promote Ca^{2+} release from plasma membrane vesicles (Delfert *et al*, 1986), there is evidence that its metabolite Ins(1,3,4,5)P_4 may directly stimulate Ca^{2+} influx. Injection of Ins(1,3,4,5)P_4 into sea urchin (*Lytechninus variegatus*) eggs resulted in the immediate elevation of the fertilisation envelope, a process known to require the presence of extracellular Ca^{2+} (Irvine & Moor, 1986). Since Ins(1,4,5)P_3 is converted to Ins(1,3,4,5)P_4 by the action of a Ca^{2+}-sensitive calmodulin-dependent 3-kinase, the direct stimulation of Ca^{2+} influx by Ins(1,3,4,5)P_4 represents a Ca^{2+}-induced Ca^{2+} entry.

It has also been argued that Ins(1,4,5)P_3 may stimulate Ca^{2+} influx indirectly through its effect on intracellular Ca^{2+} stores. The endoplasmic reticulum is functionally connected to the plasma membrane in many cell types, and extracellular Ca^{2+} has been demonstrated to flow into the endoplasmic reticulum when its store becomes depleted (Putney, 1986).

Thus, Ins(1,4,5)P_3, by depleting intracellular Ca^{2+} stores, may itself promote an influx of extracellular Ca^{2+}.

1.3.11 Diacylglycerol and protein kinase C activity

The other product of PLC-mediated hydrolysis of PtdIns(4,5)P_2 is DAG, which acts as an intracellular messenger by stimulating PKC. PKC is a family of enzymes (PKC α, β_I, β_{II} and γ) which play a key role in the initiation and modulation of receptor-mediated cellular responses through the phosphorylation of specific intracellular proteins. PKC requires Ca^{2+} and phospholipid for activity, and DAG acts to stimulate the enzyme by modulating its affinity for Ca^{2+} and phosphatidylserine (Nishizuka, 1984). Activation of PKC is associated with the Ca^{2+}-dependent translocation of the enzyme from the cytosol to the membrane, a process thought to prime the enzyme to the stimulatory effects of DAG.

It has proved extremely difficult to identify the exact substrates for PKC, although at least 18 endogenous proteins and 8 receptors have been proposed as targets for phosphorylation by PKC (Niedel & Blackshear, 1986).

Tumour-promoting agents such as phorbol-12-myristate-13-acetate (PMA) mimic the action of DAG, binding with high affinity to the regulatory region of the enzyme, strongly activating it (Nishizuka, 1984). Phorbol esters have been used extensively in the study of PKC, and, when administered concurrently with a Ca^{2+} ionophore such as A23187, can mimic the cellular effects of PLC agonists.

1.3.12 Mechanism of action in adrenocortical cells

Compared to the adrenocortical adenylate cyclase/cAMP signalling system, relatively little is known regarding the transduction of the Ins(1,4,5)P_3/DAG signal to the activation of steroidogenesis. The metabolic pathways outlined in Figure 1.8 have been shown to occur in adrenocortical cells (reviewed in Bird *et al*, 1990b). In particular, the enzymatic activities necessary for the rapid removal of Ins(1,4,5)P_3, namely 3-kinase and 5-phosphatase, have been demonstrated in both rat and bovine zg cells (Balla *et al*, 1988; Rossier *et al*, 1986), and the DAG kinase and PtdIns synthetase enzymes required for metabolism of DAG are present in adrenocortical cells (Bird *et al*, 1990b). Specific high-affinity binding sites for both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 exist in bovine adrenal cortex (Baukal *et al*, 1985; Rossier *et al*, 1986), and PKC activity has been identified in both bovine adrenal cortex membranes and rat zfr cells (Coyne *et al*, 1987; Widmaier &

Hall, 1985). Thus, the enzymatic machinery necessary for $Ins(1,4,5)P_3$ and DAG to act as second messengers is present in adrenocortical cells.

The mechanism by which $Ins(1,4,5)P_3$ and DAG stimulate steroidogenesis has been most studied in zg cells, which secrete aldosterone in response to angiotensin II via increased PtdIns turnover (see section 1.6.4). In superfused bovine zg cells, neither administration of the Ca^{2+} ionophore A23187 nor of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) alone could reproduce the steroidogenic effect of AII in full; the effect of A23187 was transient, whereas that of TPA was slower and of much lower magnitude than that of AII (Kojima *et al*, 1984). The prolonged stimulatory action of AII could, however, be reproduced by co-administration of A23187 and TPA, indicating that both sustained activation of PKC and Ca^{2+} influx are necessary for stimulation of steroidogenesis by PLC agonists (Kojima *et al*, 1984). In the same preparation, administration of nitrendipine, which blocks Ca^{2+} influx through plasma membrane Ca^{2+} channels in adrenocortical cells (Kojima *et al*, 1985a), abolished the sustained steroidogenic effect of AII but had no effect on the initial transient rise in aldosterone secretion in response to AII (Kojima *et al*, 1985b,d). Thus, the sustained stimulation of steroidogenesis by PLC agonists requires the sustained influx of extracellular Ca^{2+}.

This sustained Ca^{2+} influx may depend on phosphorylation of plasma membrane Ca^{2+} channels by PKC. At least nine different proteins were phosphorylated in response to AII in bovine zg cells (Barret *et al*, 1986b). The pattern of phosphorylation was mimicked by the combined administration of A23187 and TPA. Furthermore, the early (1 min) phosphorylation events were reproduced by A23187 but not by TPA, whereas the converse was true for the late (30 min) phosphorylation events (Barret *et al*, 1986b).

Villgrain *et al* (1984) have shown that PKC can selectively phosphorylate purified P450scc at specific serine and threonine residues. Furthermore, both P450scc and PKC were found to be present in bovine adrenocortical mitochondria. Thus, the rate limiting step in the steroidogenic pathway may be subject to regulation by PLC agonists via a mechanism analogous to that of adenylate cyclase agonists.

1.4 DISEASES OF THE ADRENAL CORTEX

Acute deficiency of adrenocortical hormones is fatal if untreated. Such deficiency may arise from bilateral adrenalectomy, or from Addisonian crisis (see below). Symptoms of glucocorticoid deficiency include muscle weakness, gastrointestinal disturbances and impaired resistance to infection. Plasma ACTH levels are elevated due to the absence of

cortisol-mediated negative feedback effects at the pituitary. Chronic deficiency of mineralocorticoids results in fluid loss, reduced plasma sodium concentration and parallel increased plasma potassium leading to hypotension. Glucocorticoid excess is characterised by fat deposition around the abdomen and neck, loss of fat around the limbs, impaired immune function, susceptibility to bruising and osteoporosis. There is frequently hypertension due to the weak mineralocorticoid effects of cortisol. Mineralcorticoid excess results in sodium retention and potassium loss with consequent hypertension and alkalosis.

The clinical manifestation of adrenocortical insuficiency is Addison's disease. Formerly a common complication in tuberculosis, Addison's disease is now more usually associated with autoimmune disorders. While plasma cortisol levels are usually low, ACTH and pituitary peptide levels are extremely high (primary adrenocortical insuficiency), leading to skin pigmentation. Treatment is by hormone replacement, both glucocorticoid (eg. dexamethasone) and mineralcorticoid (eg. fludrocortisone). Secondary adrenocortical insufficiency is caused by pituitary disease resulting in impaired ACTH secretion.

Inappropriately elevated levels of cortisol results in Cushing's syndrome. Plasma cortisol is high at all times (ie. no diurnal rhythm), and usually unaffected by dexamethasone. Causes of Cushing's syndrome include adrenocortical tumours, in which ACTH is suppressed (common in children), pituitary tumours, in which ACTH is elevated and drives the cortex (common in adults), ectopic production of ACTH (eg. from lung tumours), and possibly alcohol-related factors. Treatment includes surgical removal of tumours, if possible, or bilateral adrenalectomy with replacement therapy.

Mineralcorticoid excess associated with low plasma renin activity is termed primary aldosteronism, or Conn's syndrome. This is usually caused by an adrenocortical ademoma, and results in hypertension with hypokalemia. The exact incidence of Conn's syndrome is unknown, but it has been estimated to account for up to 1% of all cases of hypertension. Treatment is either unilateral adrenalectomy, if only one adrenal is involved, or administration aldosterone antagonists such as spironolactone.

Several genetic diseases of the adrenal cortex have been described, of which the most common is 21-hydroxylase deficiency. This results in an inability to synthesise cortisol or aldosterone, and is associated with elevated plasma ACTH and consequent adrenal hyperplasia. Since androgen synthesis is unaffected, this excessive drive to the cortex causes increased androgen secretion resulting in virilisation in females and early sexual development in males. Treatment is by replacement therapy.

1.5 ACETYLCHOLINE

1.5.1 Synthesis and metabolism

Acetylcholine (ACh), first described in 1900, was originally identified as an adrenal gland extract capable of producing a fall in blood pressure in the rabbit following removal of the hypertensive adrenaline from the extracts. ACh is now known to act as a neurotransmitter released from postganglionic parasympathetic nerve endings, parasympathetic and sympathetic ganglionic synapses, voluntary smooth muscle motor endplate and sympathetic nerve endings in the adrenal medulla. Stimulation of these systems produces a huge variety of effects, primarily affecting cardiac, visceral, ocular and endocrine tissues.

Within nerve endings, ACh is synthesised from free choline and acetyl Co-A by the action of the axoplasmic enzyme choline acetyl transferase. Choline is synthesised mainly in the liver, and taken up from the plasma into the neuron by an active mechanism. Acetyl CoA, formed in the mitochondria through the tricarboxylic acid (TCA) cycle, is impermeable to the mitochondrial membrane, and is transported from the mitochondria to the cytosol as citrate (Figure 1.9). ACh is stored in synaptic vesicles, and released on arrival of a nerve impulse by exocytosis. Free ACh within the synaptic cleft is hydrolysed by acetylcholinesterase (AChE), located within the cholinergic synaptic cleft. Although several rather non-specific cholinesterase enzymes, such as butyrylcholinesterase (BuChEe) exist, only AChE is particularly associated with nerve endings, and is much more specific for ACh. AChE is therefore a useful marker for the identification of cholinergic innervation.

In 1914, Dale classified the actions of ACh as muscarinic and nicotinic, based on the ability of the natural compounds muscarine and nicotine to reproduce the actions of ACh. This distinction subsequently led to the recognition of muscarinic and nicotinic receptor subtypes.

1.5.2 Muscarinic acetylcholine receptor subtypes

Muscarinic receptors were originally shown to be heterogeneous based on the ability of pirenzepine to discriminate between binding sites in different tissues (Hammer *et al,* 1980). Those sites exhibiting high affinity for pirenzepine (in the brain) were termed M_1, while the lower affinity receptors in peripheral tissues were termed M_2. M_2 receptors were subsequently divided into $M_{2\alpha}$ and $M_{2\beta}$ subtypes (also known as M_2(cardiac) and

FIGURE 1.9

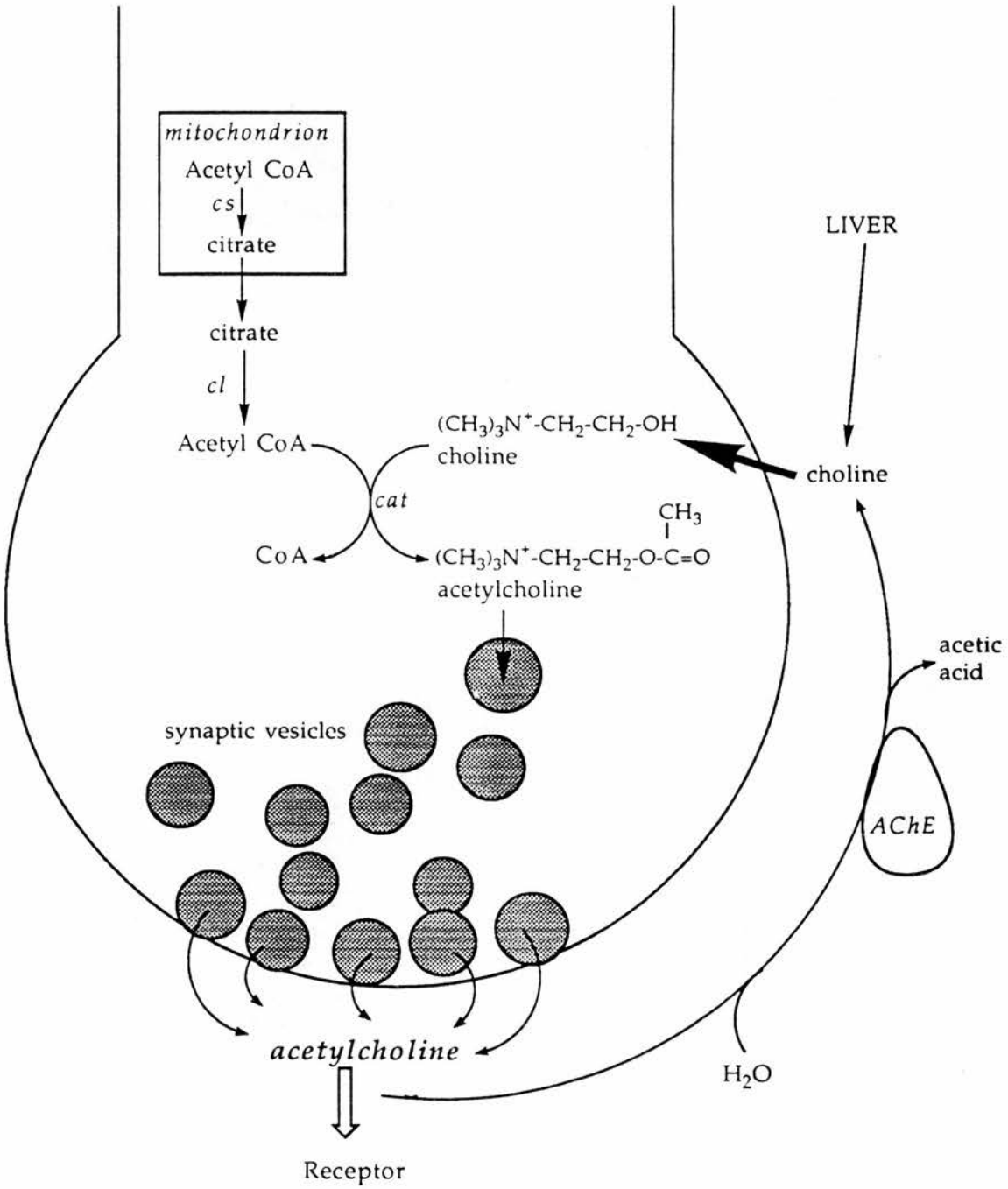

FIGURE 1.8 : Synthesis of acetylcholine in nerve terminals. Abbreviations: *AChE*, acetylcholinesterase; CoA, coenzyme A; *cat*, choline acetyltrasferase; *cl*, citrate lyase; *cs*, citrate synthase

M_2(glandular)), which resulted in some confusion until the internationally accepted nomenclature of M_1, M_2 and M_3 was adopted, where M_2 refers to the old $M_{2\alpha}/M_2$(cardiac) classification, and M_3 refers to $M_{2\beta}/M_2$(glandular) (see Table 1.4). None of the currently available specific muscarinic antagonists is completely selective for a given receptor subtype, and antagonist affinity ratios between subtypes rarely exceed ten. The pharmacological classification of muscarinic receptor subtypes therefore requires estimation of the affinity of a range of selective antagonists. Such antagonists include pirenzepine ($M_1 > M_2 = M_3$) (Hammer *et al*, 1980), methoctramine ($M_2 > M_1 > M_3$) (Melchiorre *et al*, 1987), hexahydro-sila-difenidol (HHSD) ($M_1 = M_3 > M_2$) (Fuder *et al*, 1985) and p-fluoro-hexahydro-sila-difenidol (p-FHHSD) ($M_3 > M_1 = M_2$) (Lambrecht *et al*, 1988).

Other putative receptor subtypes have been proposed. Differences in antagonist effects have been observed between hippocampal and ganglionic M_1 receptors, termed $M_{1\alpha}$ and $M_{1\beta}$ respectively (Mei *et al*, 1989). McN-A-343, an M_1-selective agonist, was much more potent in stimulating activity at $M_{1\beta}$ than at $M_{1\alpha}$ receptors. A further receptor subtype has been proposed (M_4) exhibiting high affinity for pirenzepine and AF-DX-116 (Lazareno *et al*, 1990; Garcia-Villalon *et al*, 1991). This putative M_4 receptor is distinguished from the M_1 receptor by its high affinity for himbacine (Caulfield & Brown, 1991).

Five genes encoding distinct muscarinic receptors have been cloned (m1-m5, see Table 1.4) (Bonner *et al*, 1987,1988; Peralta *et al*, 1987; Liao *et al*, 1989). The deduced amino acid sequences show high homology between the subtypes, and each exhibits seven hydrophobic regions of 20 - 30 amino acids which are assumed to be the transmembrane regions common to other G protein-linked receptors (Bonner, 1989). The antagonist binding profiles of the cloned receptors expressed in mammalian cells or *Xenopus* oocytes have shown that the m1-m4 gene products correlate with the pharmacologically-defined M_1-M_4 receptors (Buckley *et al*, 1989; Tietje *et al*, 1991). The m1, m3 and m5 receptors transfected into mammalian cells link to increased PtdIns turnover, while the m2 and m4 gene products inhibit adenylate cyclase (reviewed in Bonner (1989)). The m1, m3 and m5 receptors also appear to stimulate cAMP formation, although it is not known whether this is a direct effect on adenylate cyclase, or an effect secondary to PtdIns metabolism.

TABLE 1.4

Gene	m2	m2	m3	m4	m5
Second messenger	PtdIns	cAMP	PtdIns	cAMP	PtsIns
Receptor	M_1	M_2	M_3	M_4	-
Previous nomenclature	M_1 M_1 $M_{1\alpha}/M_{1\beta}$	$M_{2\alpha}$ $M_{2\text{-cardiac}}$	$M_{2\beta}$ $M_{2\text{-glandular}}$		
General location	brain	heart	ex/endocrine gland. Smooth muscle	?	
Specific agonists	McN-A-343	-	-	-	
Specific antagonists	pirenzepine telenzepine HHSD	meth AF-DX-116	p-FHHSD 4-DAMP HHSD		

TABLE 1.4 : Pharmacological and molecular classification of muscarinic receptors. Abbreviations: meth, methoctramine; (p-F)HHSD, (para-fluoro)hexahydro-sila-difenidol; 4-DAMP, 4-diphenylacetoxyl-N-methyl piperidine methiodide.

1.5.3 Acetylcholine effects on adrenocortical steroidogenesis

Although is has been known for some time that ACh stimulates catecholamine release from preganglionic sympathetic nerve terminals in the adrenal medulla, the possible effects of ACh in the adrenal cortex received little attention until the 1950s. Electrical stimulation of the splanchnic nerve in hypophysectomised dogs resulted in increased corticosteroid output from the adrenal cortex, although this was attributed to an indirect effect of adrenaline released into the general circulation from chromaffin cells, acting either at the level of the pituitary thereby stimulating ACTH release, or directly at the cortical cells themselves (Vogt, 1944). This possibility was eliminated by Okinaka *et al* (1952), who showed that following splanchnic nerve stimulation, venous adrenal corticosteroid concentration was elevated on the stimulated, but not the contralateral side, suggesting that the effect was independent of stimulatory agents in the general circulation.

Evidence for a direct stimulatory effect of ACh on adrenocortical cells was first provided by Rosenfield (1955), who demonstrated an increased production of 17 hydroxylated steroids in response to ACh in the isolated perfused calf adrenal gland. ACh has since been reported to stimulate corticosterone secretion from the perfused rat adrenal (Porter *et al*, 1988), as well as cortisol secretion from unpurified bovine adrenocortical cells in culture (Kawamura *et al*, 1985), and from purified bovine zfr cells both acutely isolated (Hadjian *et al*, 1982; Walker *et al*, 1990) and following primary culture (Walker *et al*, 1990). A direct effect of ACh on zg cells has also been demonstrated: ACh stimulated aldosterone secretion from the perfused rat (Porter *et al*, 1988) and frog (Benyamina *et al*, 1985) adrenal gland, and from isolated purified bovine zg cells (Kojima *et al*, 1986a).

While observations such as these clearly indicate that ACh can stimulate steroidogenesis *in vitro*, somewhat less is known regarding the possible *in vivo* role of cholinergic systems. The concentrations of ACh and acetylcholinesterase (AChE) in the adrenal cortex are about one third that of the medulla, suggesting the presence of an active cholinergic system in the cortex (Feldberg & Schild, 1934; Antopol & Glick, 1940). Indeed, AChE-positive innervation of the adrenal cortex has been described in several species, including man (see section 1.1.5). In concious hypophysectomised calves (supported by ACTH infusion), ACh infusion resulted in an increase in both adrenal cortisol secretion and plasma cortisol levels (Jones & Edwards, 1991). This effect was attributed both to a direct muscarinic effect of ACh on adrenocortical cells, and to an increase in adrenocortical blood flow, as indicated by a reduction in adrenal vascular resistance, thus enhancing the delivery of ACTH to the gland (Jones *et al*, 1991). It is

significant, given the evidence for an intra-adrenal CRH-ACTH axis (section 1.3.2), that ACh infusion also stimulated the secretion of CRH from the adrenal medulla (Jones *et al*, 1991).

Few studies have addressed the possible adrenocortical effects of ACh in man. While administration of muscarinic agonists to normal human volunteers had no effect on plasma aldosterone levels, the plasma aldosterone response to infusion of angiotensin II was inhibited by the muscarinic antagonist atropine (Stern *et al*, 1989), suggesting that cholinergic mechanisms may exert a modulatory effect on the actions of other adrenocortical agonists. Such interactions are considered further in section 1.7.

1.5.4 Mechanism of action of acetylcholine in adrenocortical cells

The mechanism by which ACh stimulates steroidogenesis in adrenocortical cells has received much attention in recent years, and seems to be similar in both zg and zfr cells. Although the effects of ACh on adenylate cyclase activity in rat adrenocortical cells have not been studied, no effect of ACh on cAMP levels has been demonstrated in bovine zfr cells (Hadjian *et al*, 1982; Walker *et al*, 1990) or bovine zg cells (Kojima *et al*, 1986a). The steroidogenic effect of ACh in bovine zfr cells was abolished in the absence of extracellular Ca^{2+} (Kawamura *et al*, 1985; Hadjian *et al*, 1982), and the cholinergic agonist carbachol stimulated an increase in both $^{45}Ca^{2+}$ fractional efflux ratio and $^{45}Ca^{2+}$ influx into bovine zg cells (Kojima *et al*, 1986a), indicating the involvement of the calcium messenger system in the response to ACh.

Subsequent studies have confirmed that ACh stimulates steroidogenesis via increased PtdIns turnover. ACh stimulated dose-dependent increases in ^{32}Pi labelling of PtdIns in bovine zfr cells (Hadjian *et al*, 1984a). The dose-dependency of the steroidogenic and ^{32}Pi-labelling effects of ACh were similar, and ^{32}Pi-labelling was found to precede any measurable increase in cortisol secretion. In primary cultures of bovine zfr cells labelled with [^{3}H]inositol, ACh stimulated the rapid formation of [^{3}H]phosphoinositols (Walker *et al*, 1990). HPLC analysis of the labelled products showed that their time course of appearance was consistent with the phospholipase C-mediated breakdown of PtdIns(4,5)P_2, ie. an early (within 5 seconds) increase in Ins(1,4,5)P_3 followed by a later increase in InsP_1, InsP_2 and Ins(1,3,4)P_3. Similar findings have been reported in bovine zg cells; although the individual phosphoinositol isomers were not separated, addition of carbachol promoted a rapid and sustained rise in [^{3}H]InsP_3 followed by slower increases in inositol bis- and monophosphates (Kojima *et al*, 1986a).

Stimulation of hormone-dependent phospholipase C by ACh would be expected to lead to an increase in $[Ca^{2+}]_i$ through the action of $Ins(1,4,5)P_3$ (Berridge & Irvine, 1989). Specific intracellular receptors for $Ins(1,4,5)P_3$ have been identified in bovine adrenal cortex (Baukal *et al*, 1985), and rapid increases in $[Ca^{2+}]_i$ in response to ACh have been reported in aquorin loaded bovine zg cells (Kojima *et al*, 1986a) and fura-2 loaded bovine zfr cells (Walker *et al*, 1991). The Ca^{2+} signal in bovine zfr cells was maintained in the presence of low (120nM) extracellular Ca^{2+}, although it decayed more rapidly than in control cells. These findings are consistent with a rapid transient release of intracellular Ca^{2+} in response to $Ins(1,4,5,)P_3$, followed by a sustained influx of extracellular Ca^{2+} maintaining the intracellular signal.

The above studies were carried out using cell populations, and were therefore unable to detect the possible occurrence of oscillations in $[Ca^{2+}]_i$ which have been shown to occur in response to phospholipase C agonists in single cells from a variety of different tissues (Fewtrell, 1993). Complex oscillations in $[Ca^{2+}]_i$ in response to angiotensin II have been reported in single fura 2 loaded rat (Quinn *et al*, 1988) and bovine (Johnson *et al*, 1989) zg cells. It is not known at present whether similar oscillatory behaviour occurs in response to ACh in zfr cells.

1.5.5 Adrenocortical acetylcholine receptors

Although a nicotinic response to ACh has been reported in the feline adrenal cortex (Rubin & Warner, 1975), this study was limited in many ways. The preparation used was an enzymatic digest of the whole cat adrenal cortex. Since nicotine is known to stimulate catecholamine secretion from the feline adrenal medulla (Douglas & Rubin, 1961), the possibility of an indirect effect of nicotine on catecholamine release from contaminating chromaffin cells (with catecholamines subsequently stimulating steroid secretion) cannot be ruled out. In addition, while nicotine was shown to stimulate corticosteroid production in this system, the effects of other cholinergic agonists/antagonists were not studied. The remaining studies which have investigated the nature of the adrenocortical cholinoceptor agree on the occurrence of a muscarinic response. ACh-stimulated steroid secretion from the calf adrenal gland was inhibited by atropine, and could not be reproduced by administration of nicotine (Rosenfield, 1955). In purified bovine zf cell suspensions, nicotine was without effect on cortisol secretion while muscarine and the muscarinic agonists carbachol, methacholine, oxotremorine, pilocarpine and arecholine significantly increased cortisol secretion (Hadjian *et al*, 1982). This steroidogenic response was inhibited by atropine, but not by the nicotinic antagonists hexamethonium and (+)-tubocurarine. The ^{32}Pi labelling of PtdIns in response to ACh in the same preparation

was also inhibited by atropine, and unaffected by nicotinic antagonists (Hadjian *et al.* 1984a). Furthermore, the increase in $[Ca^{2+}]_i$ stimulated by ACh in bovine zfr cells was inhibited by atropine (Walker *et al.* 1990). In isolated bovine zg cells, ACh-stimulated aldosterone secretion was inhibited by atropine but not by hexamethonium (Kojima *et al.* 1986a), and muscarinic stimulation of aldosterone secretion was also demonstrated in the isolated perfused frog adrenal gland (Benyamina *et al.* 1985,1987).

Specific high affinity binding sites for the muscarinic ligand [^{3}H]L-quinuclinidyl benzylate ([^{3}H]-QNB) have been demonstrated in crude membrane preparations from bovine adrenocortical tissue (Hadjian *et al.* 1981). [^{3}H]-QNB binding was potently inhibited by a variety of muscarinic antagonists, although no subtype-specific antagonists were available at that time.

The muscarinic cholinoceptor subtype mediating ACh-stimulated cortisol secretion has not been definitively classified, although preliminary evidence for the presence of the M_3 subtype in cultured bovine zfr cells has been obtained (Walker *et al.* 1990). The M_1-selective agonist McN-A-343 was without effect on cortisol secretion at concentrations up to 10^{-4}M, and although the M_1/M_3-selective antagonist HHSD dose-dependently inhibited ACh-stimulated cortisol secretion, the affinity of the antagonist was not estimated.

1.6 ANGIOTENSIN II

Angiotensin II (AII), the effector peptide of the renin angiotensin system (RAS), is produced by the actions of renin, a 42K proteolytic enzyme secreted by the juxta-glomerular cells of the kidney, and angiotension converting enzyme (Figure 1.10). The physiology of the renin-angiotensin system has been considered (section 1.3.3), and the following discussion is concerned with recent developments in the classification of AII receptors, and the adrenocortical effects of AII.

1.6.1 Angiotensin II receptor subtypes

AII receptors were originally identified by ^{125}I-AII binding studies in adrenal cortex, uterus and kidney (Lin & Goodfriend, 1970). Subsequent studies using ^{14}C- or ^{3}H-labelled AII identified AII binding sites in each of the tissues known to be stimulated by the peptide, and the binding parameters of AII in these target tissues were found to be similar (reviewed in Catt & Aguilera, 1980).

FIGURE 1.10

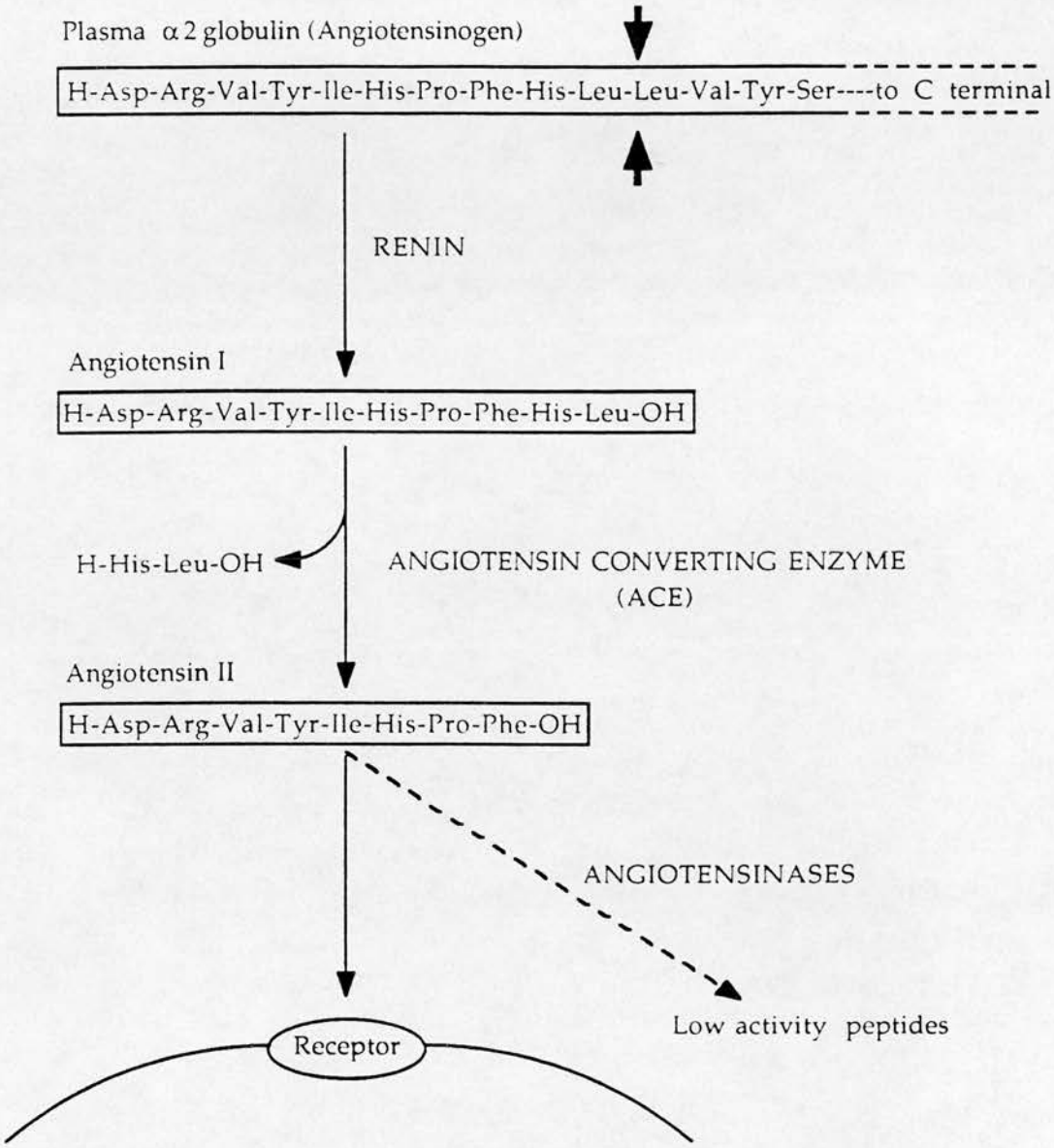

FIGURE 1.10 : Synthesis of angiotensin II from angiotensinogen. Renin, secreted by the renal juxtaglomerular cell, cleaves plasma angiotensinogen at the leu_{10}-leu_{11} bond (indicated by heavy arrows) producing the inactive decapeptide angiotensin I. Angiotensin I is further metabolised by angiotensin converting enzyme to the active hormone angiotensin II, which interacts with cell surface receptors. Angiotensinase enzymes break down angiotensin II to produce inactive/low activity peptide fragments.

Early investigations of the precise cellular location of AII receptors were confused by the demonstration that AII could enter the cell nucleus (Robertson & Khairallay, 1971). However, a variety of cell fractionation experiments confirmed the presence of AII binding sites exclusively in the plasma membrane (Devynck & Meyer, 1987). These discrepant results have subsequently been explained by the demonstration that radiolabelled AII is internalised by receptor-mediated endocytosis in adrenocortical cells (Crozat *et al*, 1986).

Initial investigations of AII receptor pharmacology studied the ability of structural analogues of AII to inhibit specific binding of ^{125}I-AII in whole cell or crude microsomal membrane preparations. The binding affinities of these peptide fragments correlated well with their ability to stimulate/inhibit AII-mediated responses in intact cells, and binding inhibition curves were invariably best fitted to single site functions (Catt & Aguilera, 1980). However, a number of observations indicated that AII receptors may be heterogeneous: (i) AII was found to use multiple mechanisms of signal transduction - in bovine adrenocortical zg cells, AII both stimulated PtdIns metabolism (Catt *et al*, 1988) and inhibited adenylate cyclase (Marie & Jard, 1983). It was not clear whether a single receptor coupled to multiple effectors, or whether distinct receptor subtypes coupled to a single effector. (ii) Differential tissue sensitivity to AII and the heptapeptide AIII ((des-asp)-AII) was reported - AIII was more potent than AII in stimulating aldosterone secretion from the adrenal cortex, whereas this order of potency was reversed when the pressor effect of the peptides was measured (Goodfriend & Peach, 1975; Campbell & Pettinger, 1979). (iii) Differential effects of structural analogues of AII between tissues were observed - $(Sar)_5$-AII and $(Pro)_3$-AII were weak agonists/partial antagonists of AII-stimulated adrenocortical steroidogenesis, but acted as full agonists in aortic smooth muscle (Capponi *et al*, 1991). (iv) Heterogeneity of AII binding sites was reported - two distinct AII binding sites were described in rat liver membranes, with affinity constants differing by some ten-fold. Binding of ^{125}I-AII to these sites was differentially affected by treatment with the sulphydryl-reducing agent dithiothreitol (DTT) (Gunther, 1984).

While studies such as these provided preliminary evidence of AII receptor heterogeneity, the lack of subtype-specific antagonists precluded a definitive classification. Unsuccessful efforts to synthesise selective AII antagonists centred around the structural modification of the peptide molecule, and it was not until 1989 that the existence of AII receptor subtypes were conclusively demonstrated using non-peptide antagonists (Chiu *et al*, 1989b).

The first non-peptide compounds reported to exhibit AII-antagonistic actions, S8307 and S8308, were described by Furukawa *et al* (1982a,b). Further study of S8308 revealed it to be a weak (ca. ten thousand times less potent than saralasin) but specific competitive antagonist of AII lacking the partial agonistic properties common to peptide analogues of AII (Chiu *et al*, 1988). An effort to synthesise more potent derivatives of this compound for possible clinical use led to the development of losartan potassium (DuP753) and analogues (Timmermans *et al*, 1991).

Losartan and related compounds were shown to potently inhibit AII-mediated responses including constriction of rabbit aorta, Ca^{2+} mobilisation in rat vascular smooth muscle cells, and to exert an antihypertensive effect in renal artery ligated rats (Chiu *et al*, 1989a). The binding affinities of these compounds correlated well with their functional antagonistic potency. However, in rat adrenocortical microsomes, losartan displaced only 80% of specific ^{125}I-AII binding, while the residual 20% was displaceable by the related compound EXP655 (now termed PD123177) (Chiu *et al*, 1989b). Autoradiographic localisation of these binding sites revealed a predominance of PD123177-sensitive sites in the rat adrenal medulla, with the majority of losartan-sensitive sites located in the outer zones of the cortex (Chiu *et al*, 1989b).

The existence of two distinct subtypes of AII binding site in the rat adrenal gland was confirmed by Chang and Lotti (1989) using the non-peptide antagonists DuP89 and WL-19, and also by Whitbread *et al*, using CGP42112A and Ex89 (Whitbread *et al*, 1989). The two binding sites were shown to be differentially affected by treatment with DTT, which potentiated binding to the losartan-sensitive site and inhibited binding to the PD123177-sensitive site (Whitbread *et al*, 1989). These binding sites were termed AT_1 and AT_2 respectively (Bumpus *et al*, 1991).

A variety of ligand binding and autoradiographic experiments revealed the presence of AT_1 and AT_2 binding sites throughout the body; both subtypes of binding site were present in rat and human adrenal (Chiu *et al*, 1989b; Whitbread *et al*, 1989; Chang & Lotti, 1989), rat uterus (Whitbread *et al*, 1989) and rat brain (Tsutsumi & Saavedra, 1991a). AT_2 sites were exclusively present in human uterus (Whitbread *et al*, 1989) and rat anterior cerebral arteries (Tsutsumi & Saavedra, 1991b), whereas cultured rat vascular smooth muscle cells contained only AT_1 sites (Whitbread *et al*, 1989). In the rabbit kidney, AT_1 sites were confined to renal medulla and glomeruli, while the outer fibrous sheath contained only AT_2 sites (Herblin *et al*, 1991). Marked species differences in the distribution of AII binding sites have been reported; rat brain containes more AT_2 than AT_1 sites, whereas rabbit brain contains predominantly AT_1 sites (Chang & Lotti, 1991).

The ratio of AT_1 : AT_2 binding sites in rabbit aorta is greater than 10:1, compared with 2:1 in rat and monkey aortae (Chang & Lotti, 1991).

An intensive effort has been made to characterise the functional significance of these distinct AII binding sites. In rat liver clone 9 cells, which express AT_1 binding sites only, AII-stimulated inositol phosphate accumulation was inhibited by losartan but not by PD123177, suggesting that in this cell line, AT_1 receptors are coupled to PLC activation (Dudley *et al,* 1990). In undifferentiated neuroblastoma NG108-15 cells, which also contain only AT_1 sites, AII stimulated a dose-dependent accumulation of $Ins(1,4,5)P_3$ and elevation of $[Ca^{2+}]_i$ (Tallant *et al,* 1991). Both responses were inhibited by losartan but not by CGP42112A (AT_2-selective antagonist). AT_1 receptors have also been shown to mediate the inhibition of ACTH-stimulated cAMP formation by AII in rat adrenal glomerulosa cells (Balla *et al,* 1991), suggesting that AT_1 receptors are coupled to both adenylate cyclase and PLC. This has been confirmed by Crawford *et al* (1992), who used 7315c tumour cells, known to contain AT_1 receptors only, to show that losartan, but not PD123177, could inhibit both AII-stimulated PLC activation via a pertussis toxin (PTX)-insensitive G protein, and AII-induced inhibition of adenylate cyclase via a PTX-sensitive G protein.

All the known effects of AII have since been shown to be mediated by the AT_1 receptor. Thus, AT_1 receptors mediate both aldosterone secretion and inhibition of ACTH-stimulated cAMP formation in rat zg cells (Balla *et al,* 1991), constriction of rabbit and rat aortae (Dudley *et al,* 1990; Chiu *et al,* 1991), growth of rat vascular smooth muscle cells (Chiu *et al,* 1991), growth of the bovine adrenocortical AC1 cell line (Natarajan *et al,* 1992), and effects of AII on noradrenergic neurotransmission in rats (Tofovic *et al,* 1991). *In vivo*, the pressor effect of AII can be abolished by losartan, which also lowers blood pressure in renal artery ligated rats (Dudley *et al,* 1990). In spontaneously hypertensive rats, the effects of AII on blood pressure are mediated by the AT_1 receptor (Tofovic *et al,* 1991), and in normal rats losartan inhibits the dipsogenic response to AII (Fregly & Rowland, 1991).

The function of the AT_2 binding site is unknown at present. AT_2 binding sites do not appear to couple to G proteins (Dudley *et al,* 1990,1991) (although see section 1.6.2). In R3T3 cells, a cell line expressing AT_2 sites only, AII had no effect on PtdIns turnover, cAMP levels, tyrosine kinase activity, arachadonic acid release, cell growth, mitogenesis or hypertrophy (Dudley *et al,* 1991). Mitogenic responses elicited by growth factors such as epidermal growth factor (EGF), bombesin of fibroblast growth factor (FGF) were unaffected by PD123177.

It had been anticipated that the discovery of AT_1 and AT_2 receptor subtypes would help to explain the large diversity of effects of AII and its use of multiple signal transduction pathways. However, the AT_2 binding site cannot yet be classed as a receptor since its function remains unknown. It is possible however that further subtypes of AII receptor exist, and indeed novel AII binding sites have been described. Binding of AII to *Xenopus laevis* cardiac membranes was unaffected by either losartan or PD123177, and CGP42112A was around three orders of magnitude less potent in displacing ^{125}I-AII in this preparation than in other tissues (Sandberg *et al*, 1991). A similar losartan and PD123177-insensitive AII binding site has been reported in differentiated murine neuroblastoma neuro-2A cells (Chaki & Inagami, 1992a). In these cells, AII promoted dose-dependent increases in cGMP formation, a response which could be blocked by nonselective peptide antagonists of AII but not by either losartan or PD123177 (Chaki & Inagami, 1992b), indicating that this novel AII receptor subtype may be coupled to guanylate cyclase activation. It is not known at present whether this receptor subtype exists in other tissues/species. However, definitive evidence for the existence of further subtypes of AII receptor has been provided by molecular biological studies.

1.6.2 Cloning of the angiotensin II receptor.

The AII receptor was first cloned by Murphy *et al* (1991) and Sasaki *et al* (1991) who used expression cloning strategies to derive complementary DNAs (cDNAs) encoding the AT_1 receptor from rat vascular smooth muscle and bovine adrenal cortex respectively. The receptor protein contained 359 amino acid residues and possessed 7 hydrophobic transmembrane regions typical of G protein linked receptors. Transfection of mammalian COS-7 cells with the receptor cDNA allowed the ligand binding characteristics of the receptor to be investigated, which were shown to be typical of native AT_1 receptors. The vascular AT_1 receptor was shown to couple to PLC activation in COS-7 cells, as demonstrated by increased inositol phosphate formation in response to AII (Murphy *et al*, 1991), and cells transfected with either the adrenal or vascular receptor cDNAs responded to AII with an increase in $[Ca^{2+}]_i$ (Murphy *et al*, 1991; Sasaki *et al*, 1991).

The human AT_1 receptor cDNA was isolated from a human liver cDNA library and shown to encode a protein 95% identical to the rat and bovine AT_1 receptor, also containing 359 amino acids and seven transmembrane regions (Bergsma *et al*, 1992). The receptor was coupled to Ca^{2+} mobilisation in *Xenopus* oocytes, and exhibited a ligand binding profile characteristic of native AT_1 receptors when expressed in COS-7 cells (Bergsma *et al*, 1992). Isolation of a genomic AT_1 receptor clone showed the entire coding region to be contained in a single exon devoid of introns (Furuta *et al*, 1992).

The availability of AII receptor cDNA probes has resulted in the identification of subtypes of AT_1 receptor which would not otherwise have been identified in the absence of specific antagonists. Two subtypes of AT_1 receptor (AT_{1A} and AT_{1B}) were isolated from a rat adrenal cDNA library. The clones were 96% identical and exhibited similar ligand binding profiles (Iwai & Inagami, 1992). Distinct AT_{1A} and AT_{1B} receptor genes were subsequently isolated from a rat genomic library and shown to both contain a 1077 base pair open reading frame encoding proteins of 359 amino acids (Elton *et al*, 1992). The AT_{1B} receptor lacked two serine residues (potential PKC phosphorylation sites) which were present in the AT_{1A} receptor. Similar AT_1 receptor subtypes have been cloned from a murine genomic library (Sasamura *et al*, 1992). No functional differences have yet been detected between AT_{1A} and AT_{1B} receptor subtypes expressed in intact cells. Although both receptor subtypes couple to Ca^{2+} mobilisation (Kakar *et al*, 1992), it is not clear at present whether this reflects an activation of PLC or a direct effect on plasma membrane Ca^{2+} channels. Furthermore, effects of the cloned receptor subtypes on adenylate cyclase activity have not been studied. In rat tissues, AT_{1A} and AT_{1B} receptor mRNA expression appears to tissue specific. AT_{1B} receptor mRNA was detected primarily in vascular smooth muscle and lung, whereas AT_{1A} receptor mRNA was preferentially expressed in adrenal gland and pituitary (Kakar *et al*, 1992). Sandberg *et al* recently reported the cloning and expression of a novel rat AII receptor subtype which was designated AT_3 (Sandberg *et al*, 1992). AT_3 receptor mRNA was most abundant in adrenal cortex and pituitary, and structural analysis revealed two additional phosphorylation sites for PKC. Compared with previously characterised AII receptors, this subtype exhibited slightly higher affinity for AII, AIII and losartan, although further data will be required to distinguish this receptor from the AT_{1A} subtype.

Recently, two independent groups succeeded in the cloning and expression of an AT_2 receptor from rat foetus (Mukoyama *et al*, 1993) and rat pheochromocytoma (Kambayashi *et al*, 1993). In both cases, a 363-amino acid protein was encoded sharing 32-33% sequence homology with the AT_1 receptor and containing seven putative transmembrane sequences. Following expression of the receptor in COS-7 cells, no effect of AII on PtdIns metabolism, $[Ca^{2+}]_i$, cAMP or cGMP levels was observed (Mukoyama *et al*, 1993), confirming the results of experiments on native AT_2 receptors (section 1.6.1). However, Kambayashi *et al* observed a dose-dependent inhibition of phosphotyrosine phosphatase activity in response to AII or to the AT_2-selective agonist CGP42112A, an effect which could be blocked by AT_2-selective antagonists but not by losartan. This represents the first clear demonstration of an AT_2 receptor-mediated response. While agonist binding to the AT_2 receptor was unaffected by non-hydrolysable

GTP analogues, the inhibition of phosphotyrosine pohosphatase activity by AII was sensitive to pertussis toxin, suggesting that this receptor may belong to a unique class of G protein coupled receptors.

In summary, pharmacological analyses have defined at least two subclasses of native AII receptor, only one of which, the AT_1 receptor, has been shown to couple functionally to intracellullar messengers. Cloning strategies have identified subtypes of AT_1 receptor, although functional differences between them have not as yet been demonstrated. The current state of AII receptor classification, based on both pharmacological and molecular studies, is outlined in Table 1.5.

1.6.3 Steroidogenic effects of angiotensin II in zfr cells

The steroidogenic effects of AII on aldosterone secretion from zg cells are well documented (see (Tait *et al,* 1980a) for review). AII binds to specific plasma membrane receptors and stimulates PLC via a pertussis toxin-insensitive G protein (Kojima *et al,* 1986b), leading to the formation of $Ins(1,4,5)P_3$, with an associated increase in $[Ca^{2+}]_i$ (reviewed in Bird *et al,* 1990b). Although AII does not stimulate cAMP formation in zg cells, it has been reported to inhibit adenylate cyclase in bovine zg cells (Marie & Jard, 1983).

Over the past fifteen years it has become clear that AII also stimulates glucocorticoid secretion from inner zone cells. AII stimulated cortisol secretion from bovine and canine adrenals (Kaplan & Bartter, 1962; Mulrow & Ganong, 1961), from bovine (Peytremann *et al,* 1973; Hepp *et al,* 1977) and human (McKenna *et al,* 1978) zf cell suspensions, and from bovine zfr cells both acutely isolated and following primary culture (Bird *et al,* 1989). Early studies using rat zfr cells produced conflicting results. High doses of AII amide stimulated corticosterone secretion in rat zfr cell suspensions, but this was subsequently shown to be due to an ACTH-like impurity in the preparation of AII employed (Hypertensin) (Tait *et al,* 1980a). Later studies failed to demonstrate any steroidogenic effect of purified AII on rat zfr cells at concentrations up to 10^{-4}M (Tait *et al,* 1980b); where stimulation of cortisol secretion by AII was demonstrated in zfr cells from other species, it occurred at low physiological concentrations similar to those found to stimulate zg cells Curiously, rat zfr cells were subsequently shown to contain AII binding sites, and exhibit a clear second messenger response to the peptide, even though it was not steroidogenic in rat zfr cells (see section 1.5.4).

TABLE 1.5

Gene	AT_{1A}/AT_{1B}	AT_2	?
Binding site	AT_1	AT_2	(AT_3)
Second messenger	PtdIns (+) cAMP (+/-)	phosphotyrosine phosphatase (-)	cGMP (+?)
Specific agonists	-	CGP42112A	-
Non-specific Antagonists	saralasin	saralasin	saralasin
Specific antagonists	losartan Ex89	PD123177	-

TABLE 1.4 : Pharmacological classification of angiotensin II receptors. Although at least three separate binding sites have been identified for angiotensin II, only the AT_1 site can presently be classed as a receptor, since the AT_2 and AT_3 sites have not as yet been definitively linked to specific cellular responses and/or second mesenger systems.

1.6.4 Mechanism of action of angiotensin II in zfr cells

Early studies on the second messenger system associated with the steroidogenic effect of AII were also confused by the use of "Hypertensin". Increases in cAMP formation in response to high concentrations of Hypertensin were attributed to the ACTH-like impurity (Tait *et al*, 1980a), and subsequent studies using purified AII failed to show any effect of AII on cAMP levels in bovine zfr cells (Tait *et al*, 1980a; Vallotton *et al*, 1981; Bird *et al*, 1988). Increasing evidence pointed to the stimulation of PLC by AII: In bovine adrenocortical zf cell suspensions labelled with ^{32}P, AII stimulated the dose-dependent incorporation of ^{32}P into phosphatidic acid and membrane phosphoinositides (Hadjian *et al*, 1984b). The dose-responsiveness of ^{32}P labelling and steroidogenesis in response to AII correlated closely. Furthermore, addition of AII to ^{32}P labelled bovine zfr cell suspensions resulted in a rapid loss of radioactivity from PtdIns(4,5)P_2 (Hadjian *et al*, 1984b). This response was abolished in the presence of the peptide AII antagonist (Sar_1-Ala_8)-AII (saralasin). Later work confirmed that AII stimulates a phosphoinositide-specific phospholipase C in bovine zfr cells. In primary cultures of bovine zfr cells labelled with [^{3}H]inositol, AII stimulated the rapid dose-dependent formation of [^{3}H]phosphoinositols (Bird *et al*, 1989). The formation of $InsP_3$ and $InsP_2$ (isomers not characterised) was shown to precede that of InsP. The predominant $InsP_3$ isomer produced on sustained (15 min) stimulation with AII was Ins(1,3,4)P_3, the only known precursor of which is Ins(1,4,5)P_3, consistent with the involvement of a PLC in the response to AII.

In rat zfr cells, AII stimulates PtdIns turnover and inositol phosphate production, but is not steroidogenic (Whitley *et al*, 1982). A similar situation appears to exist in ovine zfr cells; AII stimulated inositol phosphate accumulation in [^{3}H]inositol-labelled cells, but did not stimulate cortisol secretion (Viard *et al*, 1990). A rapid formation of $InsP_3$ was demonstrated in response to AII, which appeared to be functionally coupled to effector mechanisms, since AII also stimulated a rise in $[Ca^{2+}]_i$ (Viard *et al*, 1990). Furthermore, the cells were steroidogenically viable since ACTH was an effective stimulus to cortisol secretion. The reasons for these discrepant results in rat and ovine zfr cells are unclear.

The second messenger system involved in the stimulation of human zfr cells by AII has not been characterised in detail. However, in collagenase-dispersed suspensions of human adrenocortical cells, both A23187 and TPA were able to stimulate cortisol secretion (Laird *et al*, 1991), suggesting that inositol phosphate production and PKC activation may regulate human inner zone cells. Furthermore, in primary cultures of

human zfr cells, AII stimulated the breakdown of membrane PtdIns(4,5)P_2 without affecting cAMP levels (Ian M. Bird, Personal Communication).

1.6.5 Angiotensin II and adrenocortical Ca^{2+} mobilisation

The majority of studies on AII-induced Ca^{2+} mobilisation have been performed using zg cells. As discussed in section 1.3.9, AII stimulates a biphasic Ca^{2+} response in zg cells, the initial rise occurring as a result of Ins(1,4,5)P_3-induced release of Ca^{2+} from intracellular stores, and the sustained increase occurring due to influx of extracellular Ca^{2+}. Both phases of Ca^{2+} mobilisation are necessary for the sustained steroidogenic action of AII. Using fluorescent Ca^{2+}-binding indicators, increases in $[Ca^{2+}]_i$ have been demonstrated in cell suspensions isolated from the rat (Braley *et al*, 1986) and bovine (Capponi *et al*, 1984) zg. In single fura-2 loaded bovine zg cells, oscillations in $[Ca^{2+}]_i$ in response to AII have been reported (Johnson *et al*, 1989). An initial transient increase in $[Ca^{2+}]_i$, independent of extracellular Ca^{2+}, was followed by regular oscillations in $[Ca^{2+}]_i$, which could be abolished by removal of extracellular Ca^{2+}. Similar oscillatory behaviour has been observed in single fura-2 loaded rat zg cell (Quinn *et al*, 1988). Interestingly, rat zg cells exhibited a latency period following application of AII, the duration of which was dependent on the concentration of AII employed. Furthermore, the frequency of oscillations was related to the dose of AII used, suggesting that AII may stimulate aldosterone secretion through modulation of the frequency of $[Ca^{2+}]_i$ oscillations.

The changes in $[Ca^{2+}]_i$ occurring in zfr cells following stimulation with AII are less well documented. Rat zfr cells did not exhibit an increase in $[Ca^{2+}]_i$ in response to AII (Braley *et al*, 1986), or a release of Ca^{2+} from intracellular stores (Williams *et al*, 1981). In contrast, AII did stimulate dose-dependent increases in $[Ca^{2+}]_i$ in primary cultures of bovine zfr cells (Walker *et al*, 1991). In low Ca^{2+} medium the response declined to baseline more rapidly than in normal Ca^{2+} medium, consistent with the requirement for extracellular Ca^{2+} to sustain the Ca^{2+} signal. Evidence was also obtained that AII and ACh stimulate the release of Ca^{2+} from a common intracellular pool in bovine zfr cells (Walker *et al*, 1991). To date, the nature of the intracellular Ca^{2+} response to AII in single zfr cells is unstudied.

1.6.6 Adrenocortical angiotensin II receptors

AII receptors have been characterised in the adrenal cortex of various species by ^{125}I-AII binding inhibition studies using non-peptide antagonists. In the rat, monkey and rabbit adrenal cortex, both AT_1 and AT_2 sites are present, although the relative proportions of

binding sites are variable between species. Monkey and rabbit adrenal cortex contains mostly AT_1 sites (ratio $AT_1 : AT_2 > 9 : 1$) (Chang & Lotti, 1991), while around 20 % of AII binding sites in the rat adrenal cortex are AT_2 (Chiu *et al*, 1989b; Chang & Lotti, 1991). Conflicting data have been presented for the bovine adrenal cortex. In membranes prepared from unpurified bovine adrenocortical cells only AT_1 sites were detected (Balla *et al*, 1991). In contrast both AT_1 and AT_2 sites were detected in cultured bovine zf cells (Ouali *et al*, 1992), although the proportion of AT_2 sites was rather small. Further study will be required to resolve this discrepancy.

Adrenocortical AII binding sites have been visualised by autoradiography. In the rat adrenal cortex, ^{125}I-AII binding sites extended through the outer and inner adrenocortical zones. Around 80% of these sites were AT_1, and the remaining AT_2 sites were distributed uniformly in the zg (Chiu *et al*, 1989b). Similarly, in the rabbit adrenal cortex both the zfr and the zg were labelled with ^{125}I-AII. However, in the presence of losartan, AT_2 sites were revealed to be organised in discrete ball-like clusters throughout the cortex (Herblin *et al*, 1991). These organised patches of AT_2 sites have no known anatomical correlate, and their presence remains a mystery.

1.7 INTERACTIONS BETWEEN AII, ACh, AND OTHER ADRENOCORTICAL AGONISTS

While AII and ACh are clearly capable of stimulating steroidogenesis in both zg and zfr cells from a variety of species, an important consideration is whether such agonists can modulate the effects of other adrenocortical stimuli. It is well established that AII can inhibit adenylate cyclase in bovine and rat zg cells (Marie & Jard, 1983). This results in a dose-dependent inhibition of both basal and ACTH-stimulated cAMP production in bovine zg cell membrane fractions (Marie & Jard, 1983) and isolated rat zg cells (Balla *et al*, 1991). The physiological significance of this effect is unknown, but is may reflect a diversion of steroidogenesis from a glucocorticoid pathway to a mineralocorticoid pathway in response to AII. This is suggested also by the effects of ACTH and AII on the expression of steroidogenic genes. The activity of P450c17 determines whether pregnololone enters glucocorticoid or mineralocorticoid synthetic pathways (see section 1.2.3). P450c17 levels in the zg are very low *in vivo*, allowing the preferential synthesis of mineralocorticoids (Hornsby, 1988). ACTH is known to stimulate expression of P450c17, and this effect can be inhibited by AII in bovine, ovine and human adrenocortical cells (Rainey *et al*, 1991; Bird *et al*, 1992b; McAllister & Hornsby, 1988). This effect of AII on P450c17 expression results in an inhibiton of chronic ACTH-

stimulated cortisol secretion by AII in ovine zfr cells (Bird *et al*, 1992b), even though AII has no apparent effect on basal cortisol secretion in this system (Viard *et al*, 1990).

Interactions between AII and ACTH have been reported to occur *in vivo*. Although AII infusion in humans has no effect on plasma cortisol levels (Mason *et al*, 1976,1977), AII did produce dose-dependent increases in plasma cortisol when infused simultaneously over a low constant background infusion of ACTH (Mason *et al*, 1979). In canine species, a potentiation of AII-stimulated cortisol secretion by low concentrations of ACTH was also reported (Bravo *et al*, 1975; Slater *et al*, 1963).

1.8 SUMMARY AND AIMS OF THE THESIS

Following an introduction to the adrenal cortex and its steroid hormones, the effects of ACh and AII on adrenocortical steroidogensis have been reviewed, with particular reference to the inner zfr cell. Human zfr cells *in vitro* respond to both agonists with increased cortisol secretion via the activation of PLC, and it appears likely that ACh and AII may regulate cortisol secretion in man, *in vivo*. Although important species differences exist with respect to the adrenocortical effects of ACh and AII, the physiology of human zfr cells appears to be most closely reflected by that of bovine zfr cells in culture, and this preparation would therefore seem a good model with which to study the cellular physiology and pharmacology of these agonists in the adrenal cortex.

While it is clear that ACh stimulates cortisol secretion via muscarinic but not nicotinic receptors, the cholinoceptor subtype involved in this response has not been identified. Neither has the receptor subtype mediating AII-induced cortisol secretion been investigated. Following an initial characterisation of the bovine zfr cell culture system in Chapter 3, Chapters 4 and 5 aim to classify the ACh and AII receptor subtypes mediating the steroidogenic effects of these agonists using subtype-selective antagonists.

The remaining chapters of this thesis compare the effects of ACh and AII on phosphoinositide hydrolysis and intracellular Ca^{2+} signalling. There appear to be many similarities in the actions of ACh and AII in adrenocortical cells. For example, both agonists stimulate PLC and phosphoinositide turnover. However, it is not clear whether a common pool of phosphoinositides is metabolised in response to ACh and AII, or whether each agonist stimulates the breakdown of discrete pools of lipids. This question is addressed in Chapter 6. Finally, although ACh and AII have previously been shown to mobilise Ca^{2+} from a common intracellular pool, and to generate similar profiles of Ca^{2+} mobilisation in cell populations, the effects of these agents at the single cell level in zfr

cells is unknown. Investigations at the single cell level may reveal differences in the actions of ACh and AII not detectable in cell populations, and the abilities of ACh and AII to increase $[Ca^{2+}]_i$ in single zfr cells are compared in Chapter 7.

CHAPTER 2

MATERIALS AND METHODS

2.1 SOURCE OF MATERIALS

A231877	Sigma
Acetylcholine chloride	Sigma
Acrylamide	Sigma
ACTH (Synacthen)	CIBA
Adrenaline	Phoenix
AG1-X8 anion exchange resin	Biorad
Angiotensin II (WHO Standard 64/15)	NIBSC
Antibodies	SAPU
Amphotericin B	Flow
Atropine sulphate	Sigma
Bovine Serum Albumin (Fraction V)	ICN
8-Bromo cAMP	Sigma
Chloramine T	Sigma
Collagenase	Lorne
Cortisol-3-(*O*-carboxymethyl)oximino-2-[^{125}I]iodohistamine	Amersham
CPSR-5	Sigma
Cyclic AMP	Sigma
DEAE Sephadex A25	Sigma
Digitonin	Sigma
Earl's Balanced Salts	NBL
Fura-2 AM	Boehringer Mannheim
Fura-2 (free acid)	Boehringer Mannheim
Gelbond PGA	Sigma
Glucose	Sigma
Hams' F-10	NBL
Hydrocortisone	Sigma
Inositol	Sigma
N,N'-methylenebisacrylamide	Sigma

Myo-[^{3}H]inositol	Amersham
NADH	Boehringer Corp.
Nonidet P50	Sigma
Nylon Gauze	Henry Simon
Penicillin	Flow
Pharmalyte	Sigma
Phorbol 12-myristate 13-acetate	Sigma
Phosphate buffered saline tablets	Sigma
Pirenzepine	Sigma
Pyruvate	Boehringer Corp.
Saralasin	Sigma
Scintillation fluid	Canberra Packard
Scintillation vials	Canberra Packard
Sephadex G-10	Pharmacia
Sephadex G-50	Pharmacia
^{125}I-Sodium iodide	Amersham
Streptomycin	Flow
1,1,2-trichlorotrifluoroethane	Aldrich
Tri-n-octylamine	Aldrich
Triton X-100	BDH
X-OMAT XAR-5 X-ray film	Sigma

The following antagonists were obtained from non-commercial sources:

Hexahydro-sila-difenidol and p-fluoro-hexahydro-sila-difenidol:

Drs G. Lambrecht, E. Mutschler & R. Tacke
Johann-Wolfgang-Gothe University
Frankfurt am Main
Germany.

Methoctramine:

Prof. Dr. C. Melchioorre
Universita' Delgi Studi di Bologna
Italy.

Losartan potassium and PD123177:

Dr. Roland D. Smith
DuPont
Willmington, DE
USA.

All other chemicals were of Analar grade and obtained from BDH and Aldrich.

Addresses of Suppliers (all United Kingdom):

Aldrich	Aldrich Chemical Co. Gillingham Dorset
Amersham	Amersham International Aylesbury Bucks
BDH	BDH Thornlibank Glasgow Strathclyde
Bio Rad	Bio Rad Laboratories Watford Herts
Boehringer Corp.	Boehringer Corporation London
Boehringer Mannheim	Boehringer Mannheim Lewes East Sussex
Canberra Packard	Canberra Packard Pangbourne Berks

CIBA	CIBA Laboratories Horsham
Flow	Flow Laboratories Rickmansworth
Herny Simon	Henry Simon Stockport Cheshire
ICN	ICN Biomedical High Wycombe Bucks
Lorne	Lorne Diagnostics Bury St. Edmonds
NBL	Northumbria Biologicals Ltd. Cramlington Northumberland
NISBC	National Institute for Biological Standards & Control Potters Bar Herts
Pharmacia	Pharmacia Milton Keynes
Phoenix	Phoenix Pharmaceuticals Gloucester
SAPU	Scottish Antibody Production Unit Carluke Strathclyde
Sigma	Sigma Chemical Co. Poole Dorset

2.2 METHODS

2.2.1 Cell isolation and culture

Bovine adrenocortical zfr cells were isolated and purified from the zfr of the adrenal gland from 1- to 2- year old steers according to the method of Williams *et al* (1989). Glands were obtained from freshly slaughtered cattle (Gorgie Abattoir, Edinburgh) and transported on ice to the laboratory within 60 minutes. All work was carried out in a sterile class II laminar flow cabinet (MDH Ltd.).

After removal of the surrounding fat, the cortex was sectioned into approx. 100 μm slices using a Stadie-Riggs microtome. The first slice (capsule and zg) was discarded, while the second slice (zfr) was collected into 5 - 10 ml Earl's balanced salt solution (EBS) containing 0.2% (w/v) bovine serum albumin (BSA). 4 - 5 g of zfr tissue were routinely collected. The tissue was minced into 1 mm^2 sections, washed thoroughly with 50 - 60 ml EBS, and digested for 2 h in 50 ml EBS containing 2% (w/v) BSA and 2 mg/ml collagenase. The tissue was gently agitated at 30 min intervals during digestion, after which the resulting suspension was filtered through coarse and medium nylon gauze (250 μm and 100 μm respectively), and the cells harvested by centrifigation at 450 x g for 30 min. The cell pellet was resuspended in EBS / 0.2% BSA and filtered through fine (30 μm) nylon gauze.

Further purification was achieved by the column filtration method of McDougall *et al* (1979). The cell suspension was applied to a scintered glass Allihn funnel (100 mm x 20 mm disc / 16 - 40 μm pore size) containing 15 ml Sephadex G-10 covered by 5 ml Sephadex G-50, and encouraged through the column by gentle suction applied with a syringe. This procedure trapped intact cells within the Sephadex beads, while allowing contaminating red blood cells, cell debris and the smaller zona reticularis and glomerulosa cells to pass through. The column was washed with 20 ml EBS / 0.2% BSA, and the cells harvested by passing the resuspended gel through 30 μm nylon gauze, thus trapping the Sephadex beads while allowing the cells through. The cell suspension was centrifuged at 450 x g for 30 min and finally resuspended in 50 ml culture medium (Ham's F10 medium containing 10% (v/v) Controlled Serum Replacement No. 5 (CPSR5), penicillin (50 IU / ml), amphotericin B (2.5 μg / ml) and streptomycin (50 μg / ml). Cells were counted using an improved Neubauer haemocytometer. The isolation and purification procedure routinely yielded 50 - 100 x 10^6 cells essentially free from cell debris and contaminating glomerulosa cells (Williams *et al*, 1989).

Freshly isolated cells were either used immediately or seeded to confluence in 12- or 24-well plates at densities of 330000 or 165000 cells / ml / well respectively. For fluoresence microscopic studies, sterile glass coverslips (thickness No. 1, 22 mm diameter) were placed into 12-well plates, and cells seeded onto the coverslips at half normal density (165000 cells / ml / well). In some experiments, cells were seeded in 25 cm^2 or 75 cm^2 tissue culture flasks at densities of 3 x 10^6 cells / flask (5 ml) and 10 x 10^6 cells / flask (15 ml) respectively. In all cases, cells were maintained at 37°C in an atmosphere of 5% CO_2 in a humidified incubator (Scotlab VSL) until required. The medium overlying the cells was renewed 24 h after initial plating.

2.2.2 Preparation of zfr microsomal membranes

Crude zfr cell membrane fractions were prepared by a modification of the method of Catt *et al* (1980). 48 h after initial plating, cells cultured in 75 cm^2 flasks were washed with 10 ml pre-warmed EBS and left under 5 ml Ca^{2+}- and Mg^{2+}-free EBS. Cells were incubated at 37°C with occasional agitation for 5 min or until they had completely lifted from the bottom of the flask. Following centrifugation at 450 x g for 30 min at 4°C, cells were resuspended in 20 mM ice-cold $NaHCO_3$ (1ml / 10 x 10^6 cells), kept on ice and sonicated (20 µm amplitude, 6 x 2s). The resulting cell homogenates were centrifuged at 3000 x g for 10 min at 4°C, and the combined supernatants centrifuged at 20000 x g for 30 min at 4°C. The resulting pellet was washed with membrane buffer (20 mM TRIS / HCl, 120 mM NaCl, 0.2% BSA, pH 7.4), and finally resuspended in 1-2 ml of the same. A small aliquot was removed for protein determination.

2.2.3 Stimulation of cortisol secretion

(i) Freshly isolated cells

Freshly isolated cells (in culture medium) were centrifuged at 450 x g for 30 min and resuspended in EBS with added BSA (0.2% w/v) and glucose (0.1% w/v) (EBSBG) at a density of 330000 cell / ml. Cells were dispensed into 1.5 ml Eppendorf tubes (1ml / tube), pelleted by centrifigation at 500 x g for 3 mins in a Heraeus Christ Biofuge B, and resuspended in 1 ml EBSBG. The centrifugation step was repeated, and the cells resuspended in 0.45 ml EBSBG. Following preincubation at 37°C for 5 min, agonists were added to a final volume of 0.5 ml, and stimulation allowed to proceed for 60 min before the cells were pelleted by centrifugation and the cell - free medium recovered and stored at -20°C prior to cortisol radioimmunoassay.

(ii) Cultured cells

The basic protocol for stimulation of cultured and freshly isolated cells was similar, except that for medium changes on cultured cells, the medium was removed by aspiration and replaced with fresh medium. Cells in 12 well plates were washed twice with 1 ml EBSBG and left under 0.45 ml medium. Following preincubation for 5 min at 37°C, agonists and / or antagonists were added to a final volume of 0.5 ml. Cells were incubated at 37°C for the times indicated before the overlying medium was removed and stored at -20°C prior to cortisol radioimmunoassay.

2.2.4 Stimulation of cAMP secretion

cAMP secretion into the medium was measured in freshly isolated or cultured cells following a protocol exactly the same as described above (sections 2.2.3i/ii) except that the final incubation volume was 1 ml. At the end of the incubation, the recovered medium was split into 2 x 0.5 ml aliquots. One aliquot was stored at -20°C for cortisol radioimmunoassay, while the other was transferred to a glass tube, acidified to pH 5.0 by the addition of 5 µl acetic acid (20% v/v), and immediately acetylated by the addition of 15 µl of a 2:1 mix (v/v) of triethylamine and acetic anhydride. Acetylated samples were stored at -20°C prior to cAMP radioimmunoassay.

2.2.5 Stimulation of phospholipase C

Phospholipase C activation was determined by measuring the agonist-stimulated production of [^{3}H]phosphoinositols (in the presence of LiCl) in [^{3}H]inositol prelabelled cells. This technique allows the radioactivity associated with cellular phosphoinositide, phosphoinositol and free inositol fractions to be monitored.

(i) Cell labelling with [^{3}H]inositol

Where steady state labelling was required, cells were plated out in 12 well multiwell plates as described. 24 h after plating, the overlying culture medium was replaced with fresh medium containing *myo*[^{3}H]inositol (10 µCi / ml). Cells were incubated at 37°C for a further 48 h before use.

In some experiments, the responsiveness of cells to phosphoinositidase C agonists was studied on each of the first five days in primary culture. In this case, cells (either freshly

isolated or following specific periods of primary culture) were incubated with [^{3}H]inositol (10 µCi / ml) included in the culture medium for 5 h only before use.

Where the effect of acetylcholine or manganese stimulation on phosphoinositide labelling was investigated, cells were cultured under normal conditions for 66 h. [^{3}H]Inositol (10 µCi / ml) was added for a further 6 h, in combination with either $MnCl_2$ (1 mM) for the entire 6 h labelling peroid, or ACh (0.1 mM) for the last 1 h of labelling only. ACh stimulation was terminated by the addition of 5 µl of atropine (1 µM final concentration) to each well for 5 min.

(ii) Agonist stimulation of phospholipase C

Once labelling had been achieved, the [^{3}H]inositol containing culture medium was removed and replaced with 0.5 ml EBSBG. Cells were incubated for 15 min at 37°C after which the overlying medium was replaced with 0.45 ml fresh medium containing LiCl and inositol (both 10 mM). Following a further 15 min incubation, agonists and / or antagonists were added to a final volume of 0.5 ml, and stimulation allowed to proceed for 15 min. Stimulation was terminated by the addition of ice-cold perchloric acid (15% v/v, 250 µl / well). Wells were scraped using a 1 ml syringe stopper and the well contents transferred (with a 0.5 ml water wash) to a 1.5 ml Eppendorf tube. Following centrifugation at 3300 x g for 3 min, the [^{3}H]phosphoinositol - containing supernatant was removed to glass tubes for neutralisation. Neutralisation was achieved by the extraction of perchloric acid using a 1:1 mixture (v/v) of 1,1,2-trichlorotrifluoroethane and tri-n-octylamine (1.5 ml / tube). Tubes were thoroughly mixed and the resulting three phases separated by brief centrifugation. The neutral top phase was recovered and stored at -20°C prior to [^{3}H]phosphoinositol assay. The insoluble pelleted material recovered from the perchloric acid extracts was frozen at -20°C under 200 µl of water for assay of [^{3}H]phosphoinositides.

(iii) Freshly isolated cells

Freshly isolated cells in suspension (330000 cells / 0.5 ml) were labelled with [^{3}H]inositol for 5 h as described above (section 2.2.5i). Cells were dispensed to 1.5 ml Eppendorf tubes (0.5 ml / tube) and centrifuged at 500 x g for 3 min. Cells were resuspended in EBSBG (0.5 ml / tube) and incubated for 15 min at 37°C. Following recentrifugation, cells were resuspended in 0.45 ml EBSBG with added LiCl and inositol (both 10 mM) and incubated for 15 min after which agonists were added to a final volume of 0.5 ml. Stimulation was continued for 15 min after which 250 µl of 15% (v/v) perchloric acid

followed by 0.5 ml water were added to each tube. The perchloric acid extracts and pelleted material were then processed as above (section 2.2.5ii).

2.2.6 Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} concentration was measured in single fura-2 loaded zfr cells by fluorescence microscopy. Fura 2 acetoxymethyl ester (fura-2 AM) is taken up by cells and cleaved by intracellular esterases to produce the membrane-impermeable Ca^{2+} sensitive (free acid) form of the dye. Since fura-2 undergoes an absorption shift to shorter wavelengths on Ca^{2+} binding (see Figure 2.1), measurement of fluorescence intensities emitted at excitation wavelengths specific for the Ca^{2+}-bound and Ca^{2+}-free forms of the dye produce a fluorescence ratio directly proportional to $[Ca^{2+}]_i$, independent of the dye concentration.

(i) Loading of fura-2

In order to optimise conditions for the loading of fura-2 into the cytosolic compartment of cultured cells, the time course and concentration dependence of dye uptake was investigated. Freshly isolated cells were seeded in 25 cm^2 tissue culture flasks (3 x 10^6 cells / flask) and used 48 h later. Fura 2 AM (20 µl) or vehicle (dimethyl sulphoxide, DMSO) was added to the overlying culture medium at various concentrations for the times indicated at 37°C. Following loading, cells were washed four times with 5 ml modified Kreb's buffer (MKB) containing (mM): NaCl, 140; KCl, 4.5; $MgCl_2$, 1.0; NaH_2PO_4, 1.0; $CaCl_2$, 2.5; glucose, 5.0; HEPES, 10.0, pH 7.4, and left under 2 ml MKB. Cells were harvested using a cell scraper with a 2 ml wash, and sonicated (20 µm amplitude, 6 x 2 s) to release intracellular fura 2. 2 ml aliquots of the cell suspensions were transferred to a quartz cuvette (1cm path length) and fluorescence measurements carried out at 37°C at an excitation wavelength of 362 nm, recording at 510 nm emmission on a Perkin-Elmer model LS-5 luminescence spectrophotometer. The suspensions were stirred throughout using a magnetic microstirrer. The fluorescence values were corrected for autofluorescence obtained from cells loaded with DMSO only.

(ii) Single cell fluorescence measurements

Freshly isolated cells were seeded onto glass coverslips and used after 48-72 h. Cells were loaded with 5 µl fura 2 AM in 0.5 ml culture medium (final fura-2 concentation 10 µM) for 1 - 2 h at 37°C. Following the loading period, cells were washed with 3 x 1 ml MKB to remove extracellular fura 2. The glass coverslip was placed in a thermostated

FIGURE 2.1

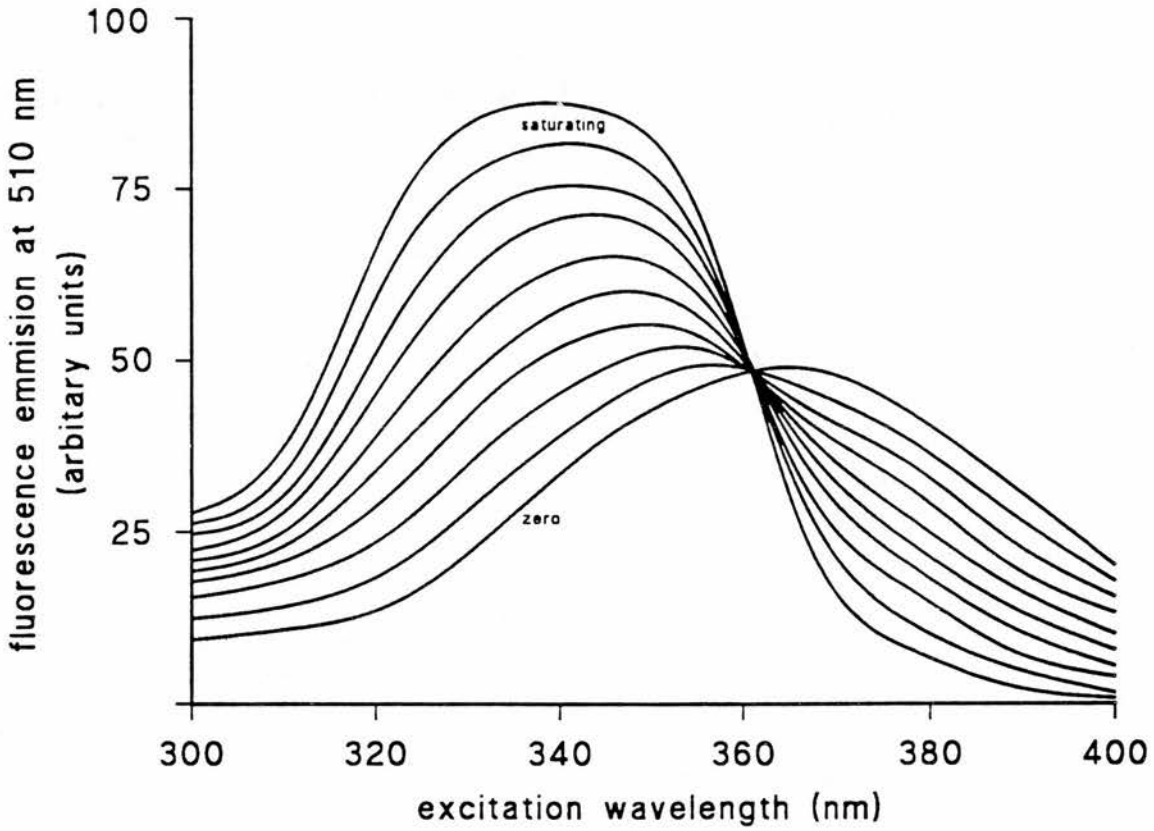

FIGURE 2.1 : Theoretical excitation-emission relationships for fura-2 free acid in solutions containing increasing concentrations of free Ca^{2+} ranging from zero to a saturating concentration (around 1 μM). This diagram shows the shift to shorter wavelengths of the dye emission maxima occurring on Ca^{2+} binding, and also illustrates the isosbestic point of fura-2 (362 nm).

superfusion chamber attached to a Nikon diaphot inverted microscope, and the cells perfused with prewarmed (37°C) MKB at a flow rate of 2 ml / min. Test substances were dissolved in MKB to achieve the stated final concentrations and delivered to the cells as continuous perfusions.

An adjustable apperture allowed the field of view to be reduced so as to encompass a single cell only. Cells were excited alternately at 340 and 380 nm, and the fluorescence intensity emitted at 505 nm at each excitation wavelength was detected by a pair of photomultipliers and the data stored on an Amstrad 2086 microcomputer. The ratio of fluorescence intensities recorded at excitation wavelengths of 340 and 380 nm was corrected for background fluorescence and analysed using software (IBM "COUNT", V3.3) developed by Dr. J. Greenwell, Dept. Physiology, University of Newcastle.

In some experiments, the fluorescence ratios were calibrated for free Ca^{2+} concentration. 0.5 ml of MKB with known concentrations of free Ca^{2+} from 50 to 800 nM (prepared as described in section 2.2.6iii) and containing fura 2 free acid (1 μM) was placed in the perfusion chamber, and the fluorescence ratio (F_{340}/F_{380}) recorded.

(iii) Preparation of Ca^{2+} / EGTA - buffered solutions

Two stock Kreb's solutions were prepared: 1) Ca^{2+}-free MKB, and 2) MKB containing 10 mM $CaCl_2$ instead of EGTA. 3ml Ca^{2+}-free MKB were placed in a quartz cuvette and fura 2 free acid added to a final concentration of 1 μM. The fluorescence value at 340 nm excitation, 505 nm emmision was recorded at 37°C using a luminescence spectrophotometer with constant stirring. This value represented the fura-2 fluorescence at zero Ca^{2+} concentration (F_{min}). The 10 mM $CaCl_2$ MKB solution was titrated into the cuvette in 5 μl volumes, and fluorescence readings (F) taken after each addition. The dye was then saturated with calcium by the addition of 100 mM $CaCl_2$ until no further increase in fluorescence was observed (F_{max}). The free Ca^{2+} concentration after each addition of $CaCl_2$ was calculated according to Grynkiewiez *et al* (1985):

$$[Ca^{2+}]\ (nM) = [\ (F - F_{min}) / (F_{max} - F)\ ] \times K_d$$

The equilibrium dissociation constant (K_d) for fura 2 was taken to be 224 nM, as previously determined (Grynkiewicz *et al*, 1985). Figure 2.2 shows an example of the Ca^{2+} calibration curve generated by this analysis.

FIGURE 2.2

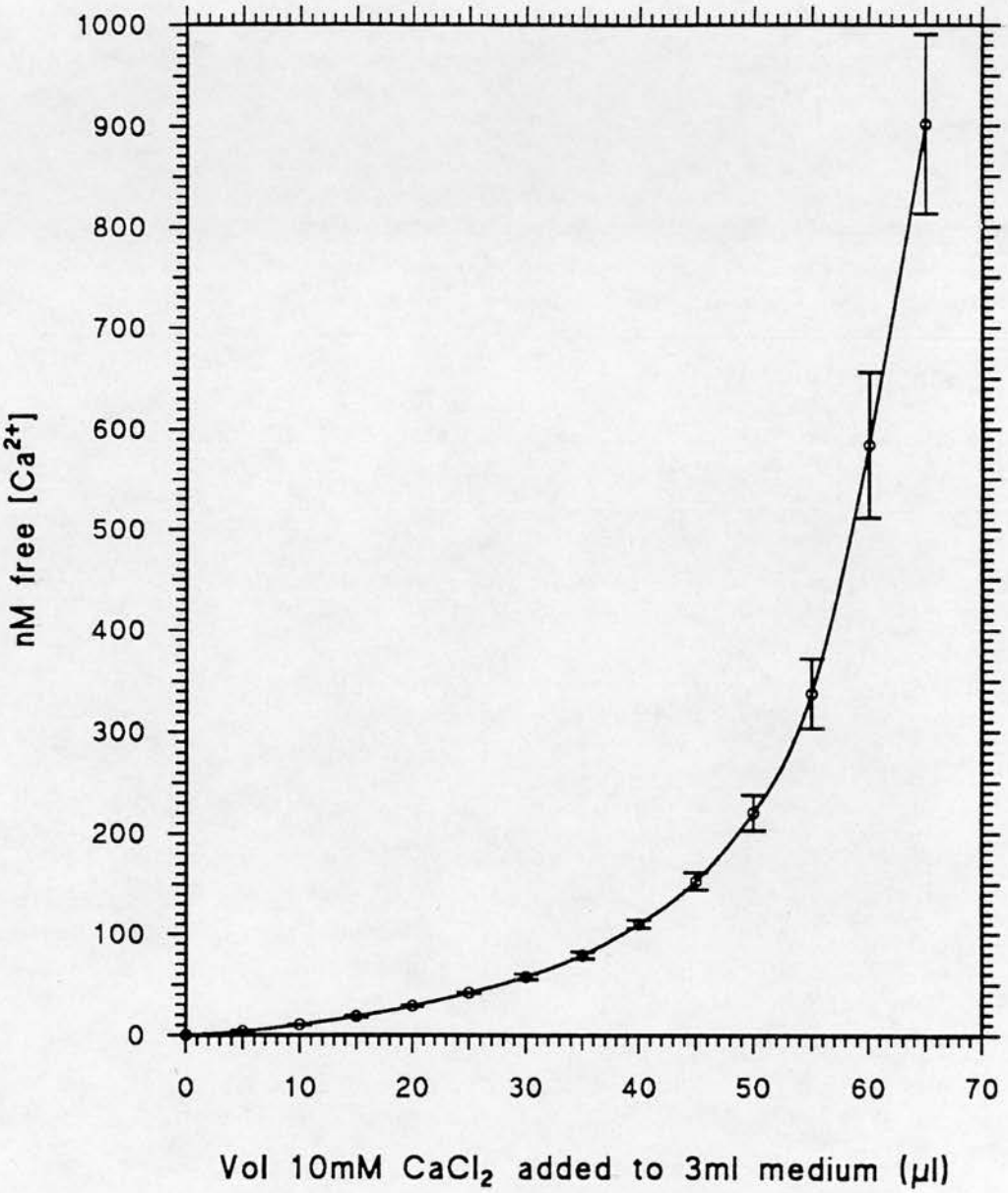

FIGURE 2.2 : Preparation of buffered Ca^{2+} solutions. Aliquots of stock Ca^{2+} solution were titrated into medium containing fura-2 free acid, and the Ca^{2+} concentration after each addition calculated from the fluorescence signal. The amount of stock $CaCl_2$ solution to be added to a given volume of medium was extrapolated from the calibration curve.

2.2.7 Measurement of ^{125}I-angiotensin II binding

(i) Preparation of ^{125}I-angiotensin

Monoiodinated ^{125}I-angiotensin II was prepared by the chloramine T method and purified by anion exchange chromatography.

10 µl each of angiotensin II (1mg / ml), Na^{125}I (1mCi, 13-17 mCi / µg I) and 0.5 M phosphate buffer (pH 7.4) were mixed together in a 1.5 ml Eppendorf tube. The reaction was initiated by the addition of 10 µl chloramine T (5 mg / ml, in phosphate buffer). After 60 s the reaction was terminated by addition of 10 µl sodium metabisulphite (5 mg / ml phosphate buffer) followed by 0.5 ml 0.1 M phosphate buffer containing 0.1% (w/v) sodium azide. The iodination mixture was applied to a 0.9 x 60 cm ion exchange column (Pharmacia K-9) containing pre-swollen DEAE Sephadex A25 in 0.1 M phosphate buffer, 0.1% sodium azide (pH 7.4), and allowed to drain into the column. 1 ml of 0.1 M phosphate buffer was then added, and allowed to drain into the column. 0.1 M phosphate buffer (0.1% azide, pH 7.4) was pumped through the column (18 ml / h) and fractions collected every 4 min. 10 µl aliquots of the fractions were counted in a gamma counter, and the peak fraction plus the 2 adjacent fractions on either side of the peak fraction pooled. The purified ^{125}I-angiotensin II was diluted 1:10 with 0.1 M phosphate buffer containing 0.1% sodium azide, 100 KI Trasylol and 0.5% (w/v) BSA, and stored frozen until required. The specific activity of the monoiodinated angiotensin II ranged from 500 - 1000 mCi / mg as determined by self-displacement radioimmunoassay (see section 2.2.9iii).

(ii) Binding of ^{125}I-angiotenin II to cultured zfr cells

Cells were plated out in 24-well multiwell plates and used for binding experiments following various times of primary culture. Cells were washed with 0.5 ml EBS / 0.1% BSA, and preincubated at 22°C for 20 min under 0.8 ml EBS / 0.1% BSA. Test substances or buffer were added in a volume of 100 µl followed by 100 µl ^{125}I angiotensin II (diluted to 10^5 cpm / 100 µl in EBS, equivalent to a final assay concentration of 0.15 nM). Non-specific binding of label was assessed as binding observed in the presence of 10^{-5}M AII. Cells were incubated for the times indicated at 22°C after which the unbound tracer was removed by rapidly washing the wells three times with 1 ml ice-cold phosphate buffered saline (0.1M, pH 7.4) (PBS). The plates were briefly blotted on absorbent paper, and 0.5 ml 1% (v/v) Triton X-100 added to each

well to solubilise the cells. The cell-bound radioactivity was recovered to plastic tubes with a 1 ml wash, and counted on a Nuclear Enterprise NE 1600 gamma counter.

(iii) Binding of ^{125}I-angiotensin II to freshly isolated cells

Freshly isolated cells were centrifuged at 450 x g for 30 min and resuspended in EBS / 0.1% BSA at a density of 165000 cells / 0.4 ml. Incubations were set up containing 400 µl EBS / 0.1% BSA, 100 µl ^{125}I-angiotensin II (10^5 cpm) and 100 µl test substances or buffer. The reaction was initiated by the addition of 400 µl of the cell suspension, and binding allowed to proceed at 22°C for the times indicated. The reaction was terminated by the addition of 3 ml ice-cold PBS and the cell-bound radioactivity trapped by immediate filtration through millipore filters (GF/C). The filters were washed with 2 x 3 ml ice-cold PBS, dried under air, and the filter-bound radioactivity assessed by gamma counting.

(iv) Binding of ^{125}I-angiotensin II to zfr cell membranes

Aliquots of membrane suspension (150 µl, approx. 100 µg protein) were incubated with ^{125}I-angiotensin II (10^5 cpm) and test substances (dissolved in membrane buffer: 20 mM TRIS / HCl; 120 mM NaCl; 0.2% BSA, pH 7.4) to a final volume of 250 µl. Incubation was for 70 min at 22°C after which the reaction was terminated by the addition of 0.5 ml ice cold washing buffer (20 mM TRIS/HCl; 120 mM NaCl, pH 7.4). Bound and free tracer were separated by immediate centrifugation at 10000 x g for 5 min. The membrane pellet was washed with 0.5 ml ice-cold washing buffer, resuspended in a further 0.5 ml buffer and recentrifuged. The resultant pellet was resuspended in 200 µl 50 mM TRIS / HCl (pH 7.4) containing 0.5% (v/v) Triton X-100 and incubated for 20 min at 4°C. The membrane suspension was centrifuged for 10 min at 10000 x g and the solubilised fractions collected for isoelectric focusing.

2.2.8 Analytical isoelectric focusing

Isoelectric focusing was performed using a Pharmacia 2117 multiphor II electrophoresis system. 115 x 240 x 2 mm slab gels of 4.85% (w/v) acrylamide containing 0.15% (w/v) N,N'-methylenebisacrylamide and 5% (v/v) pharmalyte (5.0 - 8.0) were prepared and cast onto GelBond PGA polyacrylamide gel support films. Ammonium persulphate (0.5 mg / ml of gel solution) was added and polymerisation achieved in 60 min at room temperature. Gels were applied to the cooling plate with a uniform layer of Nonidet P50 to ensure efficient heat transfer from the gel during isoelectrofocusing. The multiphor

chambers were filled with 1 M NaOH in order to improve the basic regions of the gel. Electrolyte solutions of 0.5 M acetic acid (anode) and 0.5 M NaOH (cathode) were used. Gels were prefocused for 45 min at 2000 V, 20 mA and 20 W, after which samples (30 µl) were applied to 4 x 6 mm pieces of filter paper positioned approximately 30 mm from the cathode. Calibration of the pH gradient was achieved by including standard p*I* markers (Pharmacia isoelectric focusing calibration kit 5.0 - 10.5). Isoelectrofocusing was for 4 h at 2000 V, 20 mA and 20 W.

Following the focusing period the gel was cut in half. The portion containing the membrane samples was dried (90 min at 60°C) and autoradiography performed for 24 - 48 h at -70°C using Kodak X-OMAT XAR-5 X-ray film. The portion of the gel containing the p*I* calibration markers was immersed in fixing solution (3.46% (w/v) sulphosalicylic acid, 11.5% (w/v) trichloroacetic acid) for 30 min, followed by destaining solution (67:25:8 water / ethanol / 17.5M acetic acid) for 15 min. Gels were stained in hot (60°C) staining solution (0.115% (w/v) Coomassie Blue in destaining solution) for 10 min, and left overnight in destaining solution.

2.2.9 Assay procedures

(i) Cortisol radioimmunoassay

Cortisol was measured by a modification of the method of Gray *et al* (1983), using a double antibody preprecipitate for separation of bound and free tracer.

Cortisol standards (1 to 2000 nM) were prepared by dilution of stock hydrocortisone (10 mM) in EBSBG and stored at -20°C until required. Cortisol tracer (cortisol-3-(*O*-carboxymethyl)oximino-(2-[^{125}I]iodohistamine)) was diluted in 0.1 M citrate buffer (0.2% (w/v) gelatine, pH 4.0) to 10^5 cpm / 700 µl. Antibody preprecipitate solution contained (per 500 ml citrate buffer): normal sheep serum (530 µl), reconstituted sheep anti-cortisol (650 µl), donkey anti-sheep (goat) (15 ml). The preprecicitate was stored at 4°C, and was diluted 1:2 (v/v) with 0.1 M citrate buffer before use.

Assay tubes containing 700 µl tracer solution, 100 µl standard / sample / QC and 250 µl antibody preprecipitate were incubated at 37°C for 2 h. Following centrifugation at 1800 x g for 30 min at 4°C, the supernatant was decanted and the pellets counted for 120 s in a gamma counter. Standard curves were fitted to a 4 parameter logistic model using the software package RIACALC (LKB/Pharmacia) on an IBM PC. The intra assay CV was < 10% over the range 12-2000 nM, and < 20% over the range 3-12 nM. The inter-assay

CV was < 10% over the range 12-2000 nM. Figure 2.3 shows an example of the standard curve obtained by this assay.

(ii) cAMP radioimmunoassay

cAMP was measured by a modification of the method of Harper and Brooker (1975) involving an overnight incubation with cAMP antibody followed by incubation with a second antibody to separate bound and free tracer.

Standard solutions of cAMP (0.0625 - 8.0 nM) were prepared in 50 mM acetate buffer (pH 5.0) and stored at -20°C until required. Pre-acetylated standards (acetylated as described in section 2.2.4), QCs and samples (50 µl) were added to 200 µl antibody / label mix and incubated overnight at 4°C. (Antibody / label mix consisted of 50 mM acetate buffer, 0.1% BSA, pH 5.0 containing anti-cAMP antibody (1/10000) and tracer (adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I]iodo-tyrosine, 10^5 cpm / tube)). Assay tubes were incubated at room temperature for 1 h with 100 µl second antibody preprecipitate (20 ml donkey anti- rabbit antibody and 1.5 ml normal rabbit serum / 50 ml 50 mM phosphate buffer, pH 7.4). 1.5 ml wash solution (0.15% (v/v) Brij containing 500 mg / l microcrystalline cellulose) was added and the assay tubes centrifuged at 1800 x g for 30 min at 4°C. The supernatant was decanted and the pellets counted for 200 s in an LKB/Pharmacia 1261 multigamma counter. The standard curve was constructed using a smoothed spline fit (LKB / Pharmacia RIACALC package). Intra-assay CV was < 10% over the range 0.3 - 3 nM and < 20% over the range 0.1 - 20 nM. The inter-assay CV was less than 13% over the range 0.3 - 3 nM. Figure 2.4 shows an example of the cAMP standard curve generated using this assay.

(iii) Angiotensin II radioimmunoassay

Specific activity of ^{125}I-angiotensin II prepared by the chloramine T method was established by the self-displacement method. Separation of bound and free radiolabel was achieved by adsorption of unbound tracer onto activated charcoal.

Incubations consisted of 100 µl anti-angiotensin II antibody (1 / 300000 final dilution), 100 µl ^{125}I-angiotensin II and 50 µl standard angiotensin II (0.02 - 10.24 ng / ml). All reagents were prepared in 0.1 M phosphate buffer, 0.1% (w/v) BSA, pH 7.4. Tubes were incubated overnight at 4°C after which 0.5 ml charcoal solution (0.1 M phosphate buffer containing 0.5% activated charcoal, 0.05% dextran 0.03% gelatine, pH 7.4) was added. Tubes were centrifuged at 1800 x g for 30 min at 4°C, the supernatant aspirated and the

FIGURE 2.3

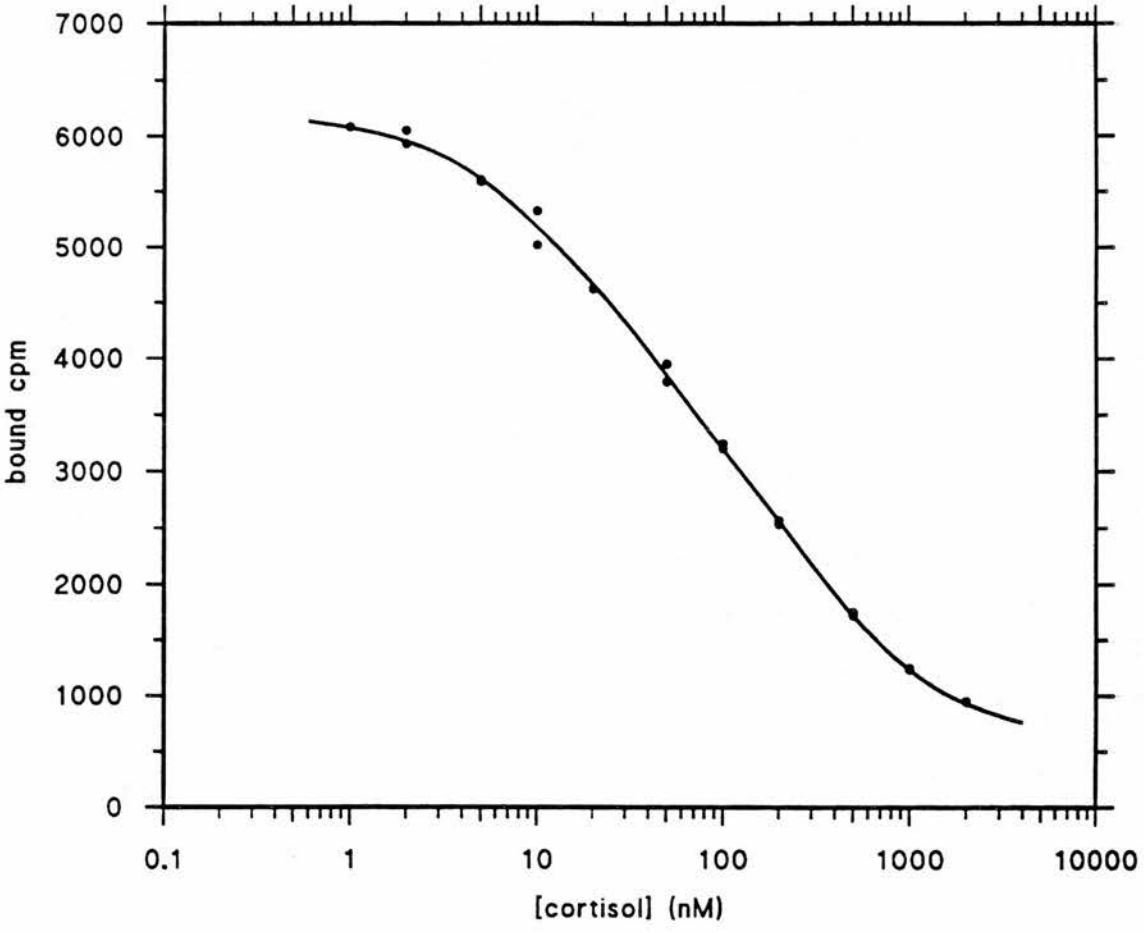

FIGURE 2.3 : Representative standard curve for the ^{125}I radioimmunoassay of cortisol. Standard solutions of cortisol (in EBS/BSA/glucose, 1 - 2000 nM) were assayed in duplicate, and the inhibition curve fitted to a four parameter logistic equation. Label binding in the absence of cortisol was typically 60-65% of total.

FIGURE 2.4

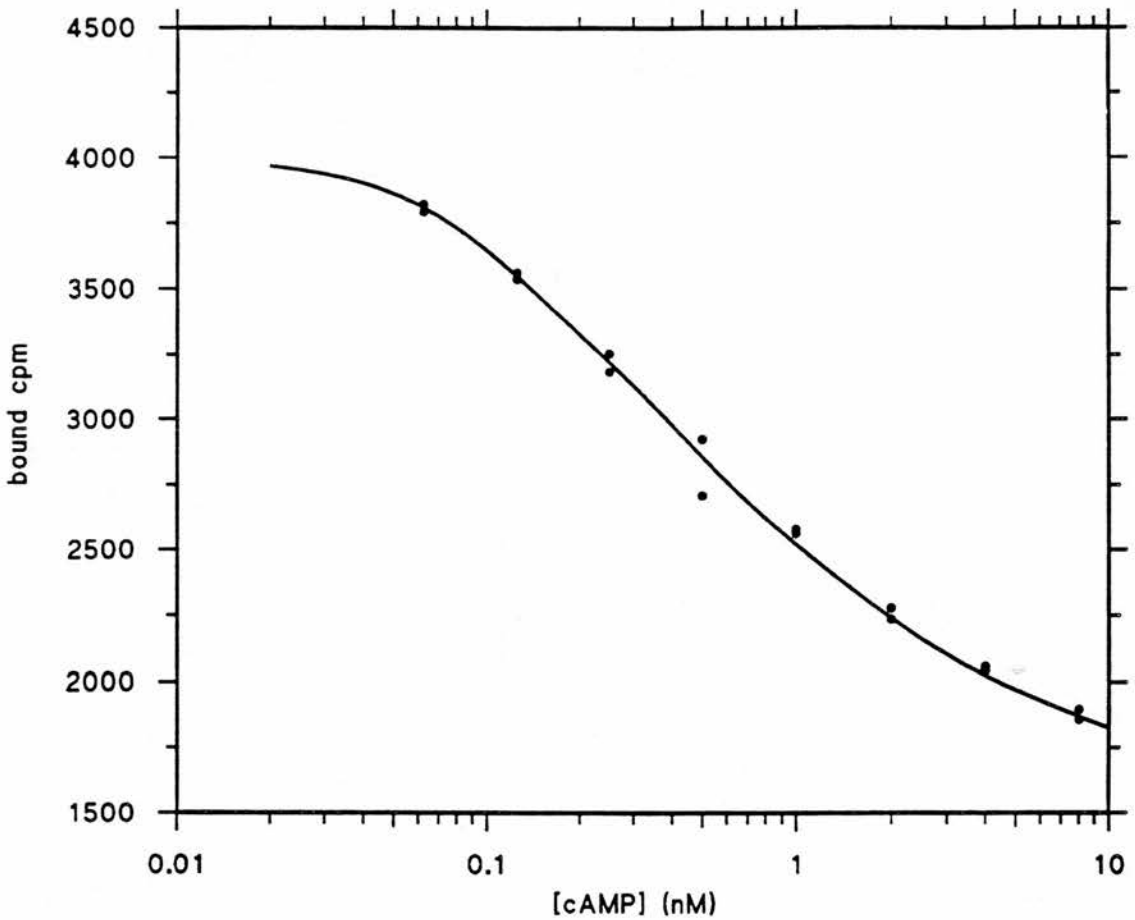

FIGURE 2.4 : Representative standard curve for the ^{125}I radioimmunoassay of cAMP. Standard solutions of cAMP (pre-acetylated in EBS/BSA/glucose, 0.0625 - 8 nM) were assayed in duplicate, and the inhibition curve fitted using a smoothed spline fit. Label binding in the absence of cortisol was typically 55-65% of total.

FIGURE 2.5

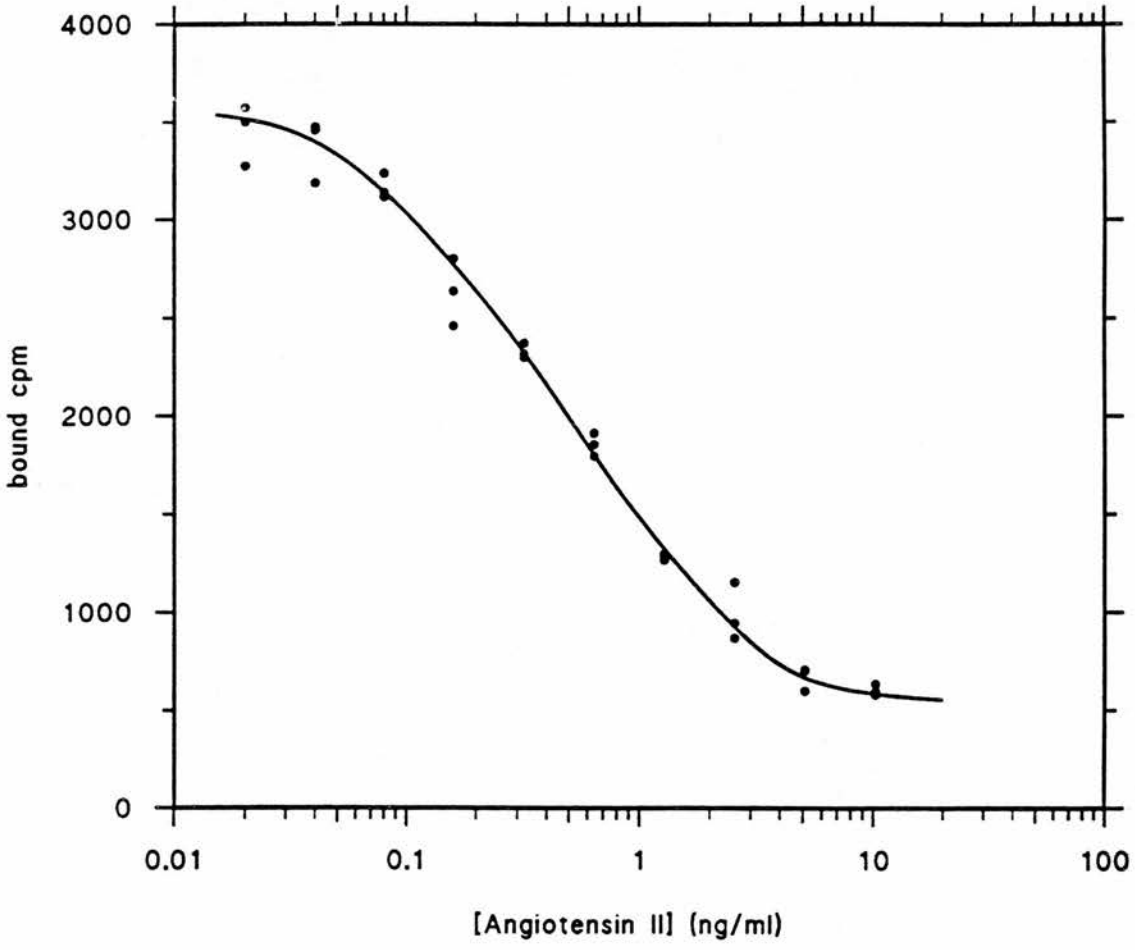

FIGURE 2.5 : Representative standard curve for the self-displacement of ^{125}-I angiotensin II by unlabelled (cold) angiotensin II. Standard solutions of angiotensin II (0.02 - 10.24 ng/ml, in phosphate buffer) were assayed in triplicate. The specific activity of the label calculated from the standard curve ranged from 500 - 1000 mCi/mg AII.

charcoal pellets containing free (unbound) tracer counted for 120 s in a gamma counter. A typical standard curve is shown in Figure 2.5

(iv) Total [^{3}H]phosphoinositol assay

Aqueous neutralised samples recovered from perchloric acid extracts were thawed and EDTA added to a final concentration of 1 mM. Samples were loaded onto individual columns of AG1-X8 anion exchange resin (0.25 ml) and the columns washed twice with 4 ml water. The combined eluates from loading and washing (containing free [^{3}H]inositol) were collected into a 20 ml scintillation vial. Bound [^{3}H]phosphoinositols were then eluted directly into fresh scintillation vials with 1 M ammonium formate / 0.1 M formic acid. (2 x 2 ml). Following addition of scintillation fluid (10 ml), radioactivity was determined by counting on a Canberra Packard 1900CA liquid scintillation counter. Samples were counted for 10 min each, or to an error of < 1%.

(v) [^{3}H]phosphoinositide assay

The insoluble pellets recovered from the perchloric acid extracts were broken up by vortexing and dissolved by the addition of 750 µl $CHCl_3$ / MeOH / conc. (11.7M) HCl (100:200:1 v/v) followed by 250 µl each of $CHCl_3$ and HCl (both 0.1 M). The resulting aqueous and organic phases were separated by centrifugation at 20 x g for 5 min, and 400 µl of the lower [^{3}H]phosphoinositide - containing phase recovered to a scintillation vial. The solvent was removed under air using a Techne sample concentrator and 3 ml scintillation fluid added. Radioactivity was determined by scintillation counting.

(vi) Protein assay

Protein content of wells, isolated cells and membrane fractions was determined by the method of Bradford (1976), adapted for use on the Cobas Fara (Roche) centrifugal analyser. Samples were washed with 2 x 1 ml 0.9% (w/v) saline, solubilised in 1% (v/v) Triton X-100 and frozen at -20°C until required. Samples were diluted 1 : 10 with water and assayed against a standard of BSA made up in 0.1% Triton X-100.

(vii) Cellular lactate dehydrogenase (LDH) assay

Cells were washed twice with 1 ml EBSBG and resuspended in 0.45 ml intracellular buffer (mM: KCl, 120; NaCl, 20; $MgSO_4$, 4.18, EGTA, 1; HEPES, 20, pH 7.4). Cells were permeabilised by the addition of digitonin (final concentration 50 µM) for 15 min at

37°C after which cells were centrifuged at 3000 x g for 3 min and the cell-free supernatant decanted and stored at 4°C prior to assay. Samples were stable for several days at 4°C but LDH activity was reduced on freezing.

LDH activity was assayed by a modification of the method of Brydon and Smith (1973) adapted for use on the Cobas Fara centrifugal analyser. Stock NADH (0.2 mM) solution and stock sodium pyruvate (7.7 mM) solutions were prepared in 0.1 mM KH_2PO_4, pH 7.4. 200 µl NADH solution and 10 µl sample were incubated in each rotor cuvette at 37°C. Blank absorbances were read at 340 nm, and stock pyruvate solution (25 µl) added to initiate the reaction. Absorbance changes were read at 10 s intervals for 100 s. LDH activity was determined from the initial rate of decrease of absorbance and expressed as LDH activity units.

2.2.10 Analysis of data

Unless otherwise stated in the figure legends, data points represent the mean of triplicate determinations. Where data have been combined from several experiments, the mean ± standard error of the mean (s.e.m.) is presented. Otherwise, where single representative experiments are shown, errors are expressed as the standard deviation of the mean (SD). Statistical differences between mean values were assessed using unpaired Student's t test. Where data were transformed, statistics were preformed on the raw (untransformed) data. Transformed data were also analysed using the Mann-Whitney rank sum test. In all cases, a value of $p<0.05$ was considered significant.

(i) Analysis of radioligand binding data

For analysis of ^{125}I-angiotensin II binding to cultured cells, AII receptor number and affinity were estimated from the inhibition of equilibrium bound ^{125}I-AII by unlabelled AII. IC_{50} values for AII obtained from inhibition curves were converted to dissociation constants (K_i) by application of the Cheng-Prusoff transformation (Cheng & Prusoff, 1973):

$$K_i = IC_{50} / (1 + (L / K_D))$$

where K_i = the equilibrium dissociation constant for the inhibitor, IC_{50} = the concentration of inhibitor reducing the binding of label to 50%, L = the concentration of label, and K_D = the label dissociation constant. Assuming that the dissociation constants for ^{125}I-AII and cold AII are similar, the above equation reduces to:

$$K_D = IC_{50} - L$$

The value of K_D thus derived was used to calculate the total number of receptors present by use of the Langmuir equation:

$$b = B_{max}.L / (L + K_D)$$

where b = the amount of label bound at equilibrium, B_{max} = the total receptor concentration and L and K_D are the label concentration and equilibrium dissociation constant respectively. The validity of the above approach was confirmed by analysing inhibition curves as for saturation analysis. At each concentration of inhibitor (unlabelled AII), the specific activity of the label was corrected to take into account dilution of ^{125}I-AII by cold AII :

$$SA' = SA.L / (L + I)$$

where SA and SA' are the original and corrected specific activity respectively, and L and I are the total concentrations of labelled and unlabelled AII. SA' was then used to calculate the total binding of AII (labelled and unlabelled) at each concentration of total AII, and the binding parameters obtained from the resulting saturation curve following linearisation by Scatchard analysis (b/L *vs* b). The estimates of K_D and B_{max} obtained by this alternative method agreed well with those obtained from K_i values.

(ii) Analysis of functional data

For analysis of antagonist inhibition curves, data were fitted to a three parameter logistic function to provide estimates of ID_{50} values, and ID_{50} values quoted in the text are the mean of three such determinations from separate cell preparations.

In order to obtain estimates of dose response parameters, data were fitted using the function:

$$y = R_{max} / (1 + (EC_{50} / x)^P)$$

where R_{max} = maximum response, EC_{50} = agonist concentration producing 50% of the maximum response and P = slope factor. Dose response curves in the presence and absence of antagonist were tested for parallelism according to Kenakin (1987), and

antagonist pA_2 values ($-\log_{10}$ antagonist dissociation constant) estimated according to Arunlakshana and Schild (1959):

$$\log_{10}(r-1) = n\log_{10}[B] + pA_2$$

where r is the dose ratio observed at antagonist concentration [B], and n is the slope of the Schild regression. pA_2 was obtained by extrapolation of the line $\log_{10}(r-1)$ *vs* $\log_{10}(B)$. (Further details regarding the use of Schild analysis for the estimation of competitive antagonist affinity are given in Chapter 4). The confidence limits of pA_2 were calculated as:

$$pA_2 \pm ts(1/n + (X)^2 / S(B - X)^2)^{1/2}$$

where t = Student's t value, s is the square root of the mean sum of squares about regression, n is the number of data points and X represents the mean value of log[B]. Where the slope of the Schild regression (n) was not significantly different from unity, pA_2 was calculated using a Shild regression with slope constrained to unity (Mackay, 1978). The confidence limits associated with this estimate were calculated as:

$$pA_2 \pm ts / (N)^{1/2}$$

CHAPTER 3

CHARACTERISATION OF THE DAY-BY-DAY RESPONSIVENESS OF CULTURED BOVINE ZONA FASCICULATA/RETICULARIS CELLS TO ACETYLCHOLINE AND ANGIOTENSIN II

3.1 INTRODUCTION

Cultured adrenocortical cell preparations have been widely used for the study of agonist-stimulated steroidogenesis. The advantages of cell culture systems over other *in vitro* adrenocortical preparations have been discussed in section 1.1.6. While zona glomerulosa cells from a variety of species have routinely been purified and cultured in the past, few investigators have established purified cultures of inner zone cells. Although purified rat inner zone cell preparations have previously been widely studied, as discussed in section 1.6.4, rat zfr cells do not respond steroidogenically to angiotensin II, and are therefore of limited use as a model for the human adrenal cortex. Bovine adrenocortical zfr cells, which model more closely the human adrenal cortex, have only recently been purified and cultured; the majority of investigators have used either acutely isolated unpurified cells from bovine adrenal cortex slices (Hepp *et al*, 1977), or unpurified cultured cells from bovine inner adrenocortical zones.

Williams *et al* (1989) used the column filtration method originally devised by McDougall *et al* (1979) for the purification of rat adrenocortical cells, to establish a primary culture system of bovine zfr cells. Compared with freshly isolated cells, cells which had been maintained in primary culture for three days showed better preservation of ultrastructure, elevated cellular lipid content and enhanced responsiveness to ACTH (Williams *et al*, 1989). Other workers using bovine zfr cells have observed a similar increase in steroidogenic responsiveness following primary culture. Kawamura *et al* (1984) found that cells were maximally responsive to ACTH following 48-72 h of culture, after which their sensitivity decreased. A similar culture time-dependent increase in cellular responsiveness to catecholamines has been reported: freshly isolated cells were unresponsive to adrenaline, but exhibited a progressively increased response to this agent following primary culture (Walker *et al*, 1988). If cultured cells are to be used as an

experimental model, knowledge of such changes in responsiveness are essential. The aim of this chapter was to characterise the day-by-day responsiveness of the cell culture system to AII and ACh with respect to steroidogenesis and second messenger generation, in order that subsequent experiments could be performed at times of maximum cellular responsiveness.

3.2 RESULTS

3.2.1 Steroidogenic and second messenger responses to angiotensin II and acetylcholine

Bovine zfr cells were prepared by collagenase digestion as described in section 2.2.2ii. Immediately following isolation (day 1), and following 24, 48, 71 and 96 h in primary culture (days 2 - 5), cortisol secretion (over 1 h) and [^{3}H]phosphoinositol production (over 15 min) were measured in response to AII (0.1 μM) (Figure 3.1, upper panel). This concentration of AII is known to maximally stimulate cortisol secretion from bovine zfr cells (Bird *et al*, 1989) Results are expressed as n-fold stimulation ratios to allow data to be combined from several different experiments. Freshly isolated cells secreted cortisol in response to AII. Following 24 h in primary culture (day 2), the cortisol secretory response to AII was significantly reduced, compared to the response of freshly isolated cells ($p<0.05$). Steroidogenic responsiveness to AII increased to reach a maximum on day 3 ($p<0.01$), before declining on days 4 and 5.

The day-by-day changes in [^{3}H]phosphoinositol production in response to AII did not parallel the changes in cortisol secretion. AII-stimulated [^{3}H]phosphoinositol production was significantly increased on day 2 compared to the response of freshly isolated cells ($p<0.01$), and reached a maximum on day 3 ($p<0.01$) before declining thereafter.

The steroidogenic responsiveness of zfr cells to a maximally effective concentration of ACh (0.1 mM, Walker *et al*, 1990) over culture days 1 - 5 is shown in figure 3.1 (lower panel). As observed for the response to AII, cells were consistently less responsive to ACh on day 2 than on day 1 ($p<0.05$). In two out of three experiments, the cortisol response to ACh was completely absent on day 2. Maximum steroidogenic responsiveness to ACh occurred on day 3, after which cells became progressively less responsive to ACh, declining to a minimum on day 5.

[^{3}H]Phosphoinositol production in response to ACh increased over the first three days in culture ($p<0.05$), reaching a maximum on day 3 before declining on days 4 and 5.

3.2.2 Day-by-day changes in protein levels/LDH activity

In order to ascertain whether changes in cell number could account for the observed changes in steroidogenic responsiveness, the protein content of wells was measured on each experimental day. As shown in Figure 3.2 (upper panel), the protein content of the

FIGURE 3.1

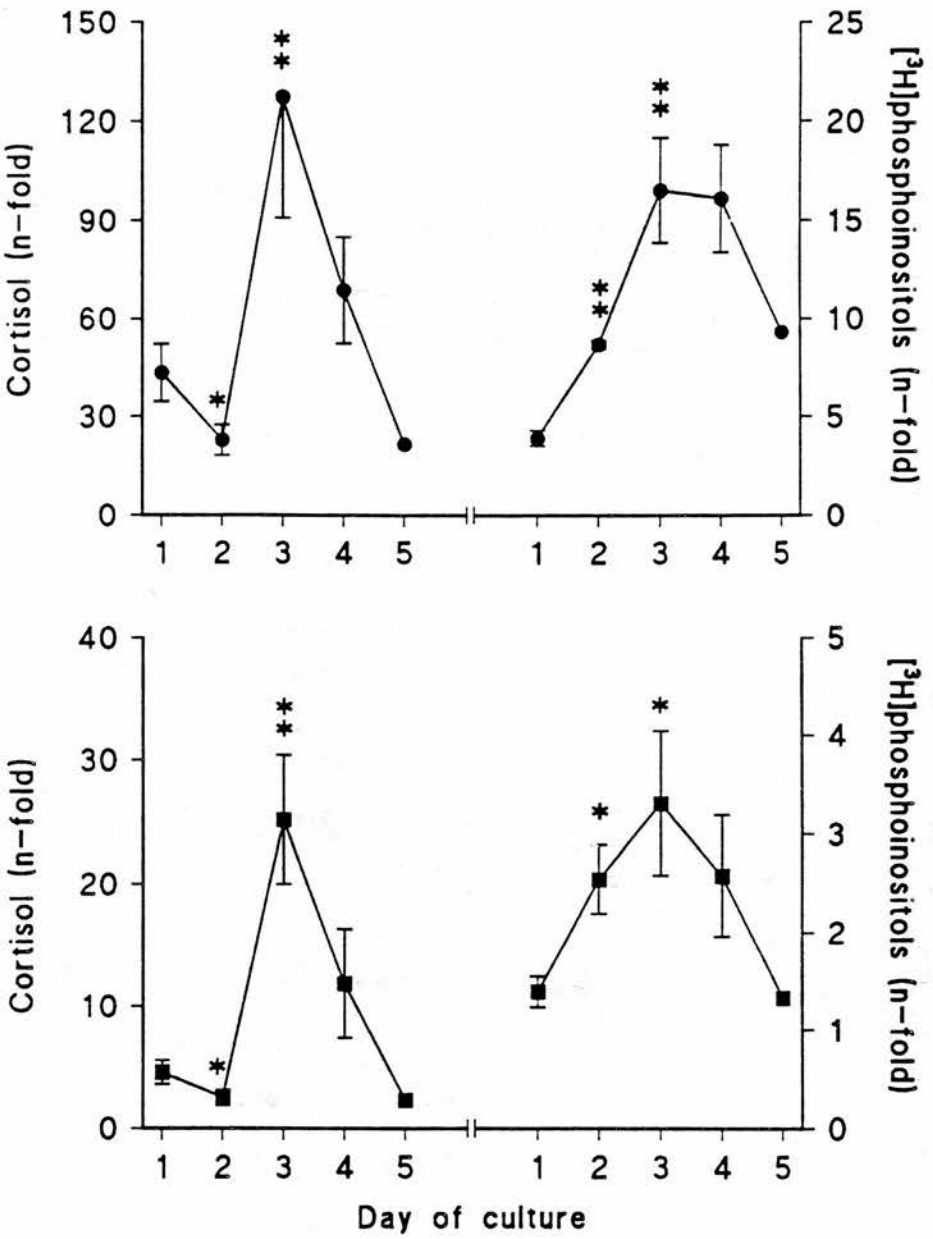

FIGURE 3.1 : Cortisol secretory response and phospholipase C response (generation of [^{3}H]phosphoinositols) of bovine zfr cells stimulated with (Top panel) angiotensin II (0.1 μM) or (Bottom panel) acetylcholine (0.1mM) on day of isolation (day 1) and on successive days in primary culture (days 2-5). Results are expressed as n-fold stimulation ratios, and show the mean ± s.e.m. of triplicate determinations from 3 separate cell preparations. Mean basal values for cortisol secretion were 6.40, 1.25, 3.25, 5.60 and 2.75 pmol/h/100 μg protein for days 1-5 respectively, and mean basal [^{3}H]phosphoinositol production was 345, 657, 723, 346 and 321 dpm/15min/100 μg protein respectively. Results significantly different from that of the previous day are indicated at *$p<0.05$ and **$p<0.01$.

FIGURE 3.2

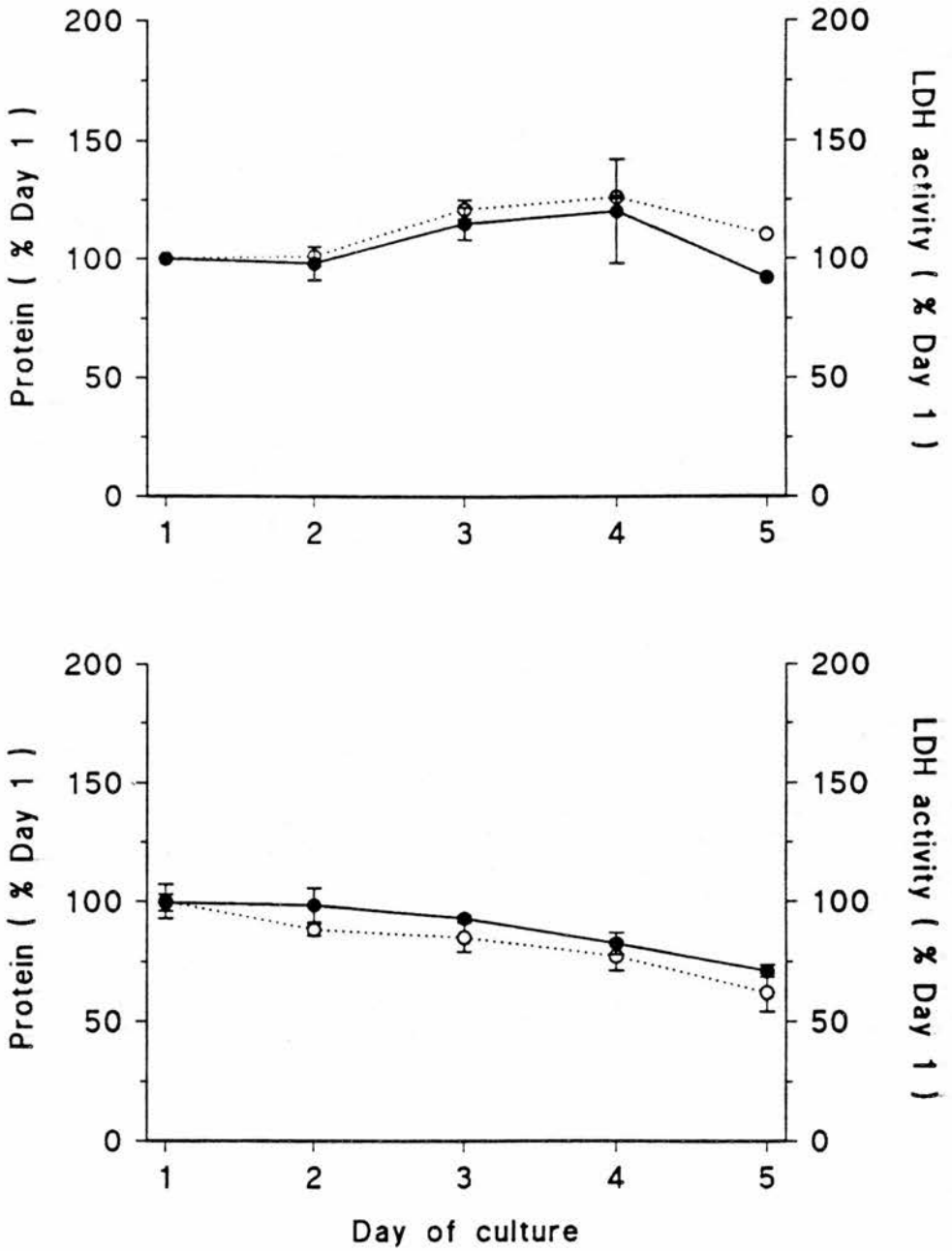

FIGURE 3.2 : Cellular protein content (solid line) and LDH activity (broken line) of bovine zfr cells on day of isolation (day 1) and on successive days in primary culture (days 2-5). Cells were cultured plated down in 12 well culture plates (Top panel), or in suspension culture in bacterial petri dishes (untreated for cell culture) (Bottom panel). Results are normalised to a percentage of the control (day 1) levels, and show the mean ± s.e.m. of triplicate determinations from 3 separate cell preparations.

wells did not vary by more than 20% over the period of study. In addition, LDH activity released from digitonin-permeablised cells was assayed as an independent measure of cell number. The levels of LDH activity on each experimental day were in close agreement with protein levels.

3.2.3 Day-by-day responses of cells cultured in suspension

The possibility that the process of cell adhesion to culture dishes may affect cellular responsiveness and give rise to the reduced steroidogenic responses to AII and ACh observed on day 2 was investigated using cells maintained in suspension culture. Freshly isolated cells were maintained in bacterial petri dishes (untreated for cell culture). Under these conditions cells did not plate down, and could be loosened from the plate by gentle scraping with a cell harvester. The day-by-day changes in protein levels and LDH activity under conditions of suspension culture are shown in Figure 3.2 (lower panel). Protein levels declined slightly over the first 5 days of culture, reaching 75% of the day 1 value after 96 h. As was observed for plated cells, protein content was closely parallelled by LDH activity on each experimental day.

Cells cultured in suspension were generally less responsive to AII and ACh than were plated cells, although qualitatively similar results were obtained in each case (Figure 3.3). For both AII and ACh, steroidogenic responsiveness was markedly reduced on day 2 compared to the response of freshly isolated cells, and peak responsiveness occurred on day 3. The [^{3}H]phosphoinositol responses to AII and ACh increased steadily from day 1 to day 4, before declining on day 5.

3.2.4 Steroidogenic and second messenger responses to ACTH and adrenaline

The reduced cortisol secretory responses to AII and ACh observed on day 2 of primary culture appeared to contradict previous studies in this system with other agonists which indicated that cellular responsiveness to ACTH and adrenaline was increased following 24 h of culture. For comparison, the day-by-day changes in cortisol secretion and cAMP formation in response to these agents are shown in Figure 3.4.

Cortisol secretion in response to ACTH increased on each successive day in culture reaching a maximum on day 4, and declined thereafter (Figure 3.4, upper panel). The increased responsiveness to ACTH was associated with increased formation of cAMP over days 1 to 4.

FIGURE 3.3

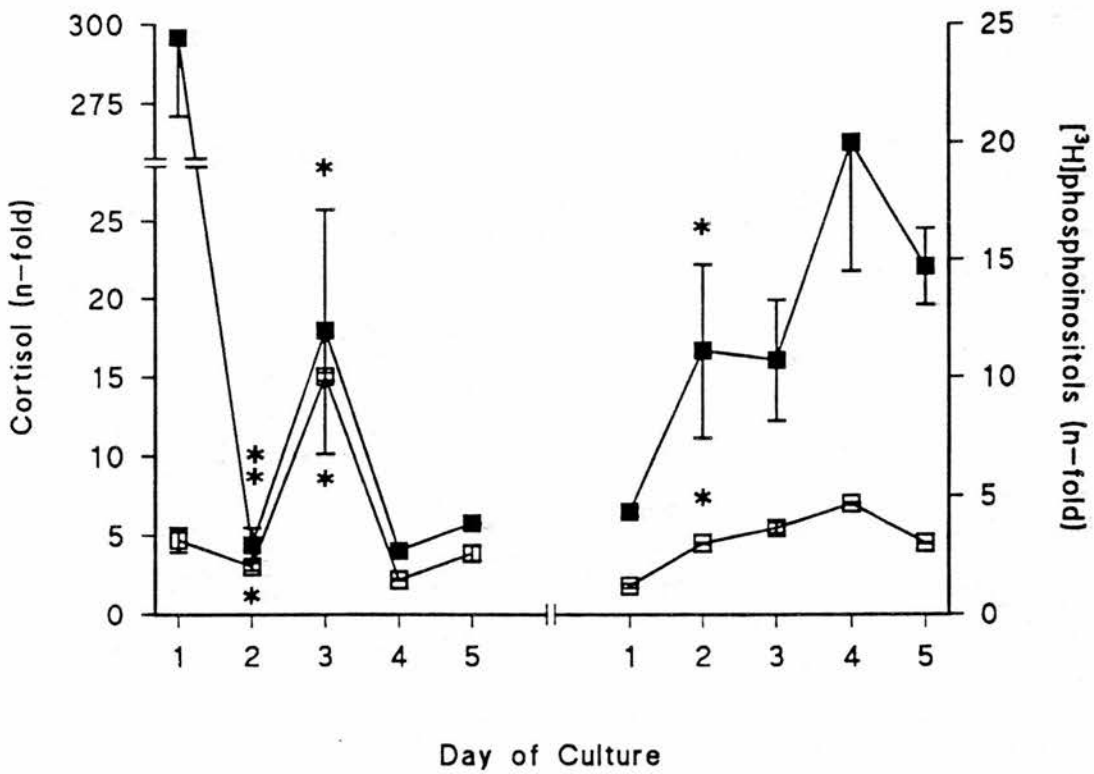

FIGURE 3.3 : Cortisol secretory response and phospholipase C response of bovine zfr cells stimulated with (■) angiotensin II (0.1 μM) or (❑) acetylcholine (0.1 mM) on day of isolation (day 1), and on successive days after maintenance in primary culture (days 2-5). Cells were maintained in suspension culture in bacterial petri dishes. Data points are the mean ± SD of triplicate determinations from a single representative experiment. Qualitatively similar results were obtained in a second experiment.

FIGURE 3.4

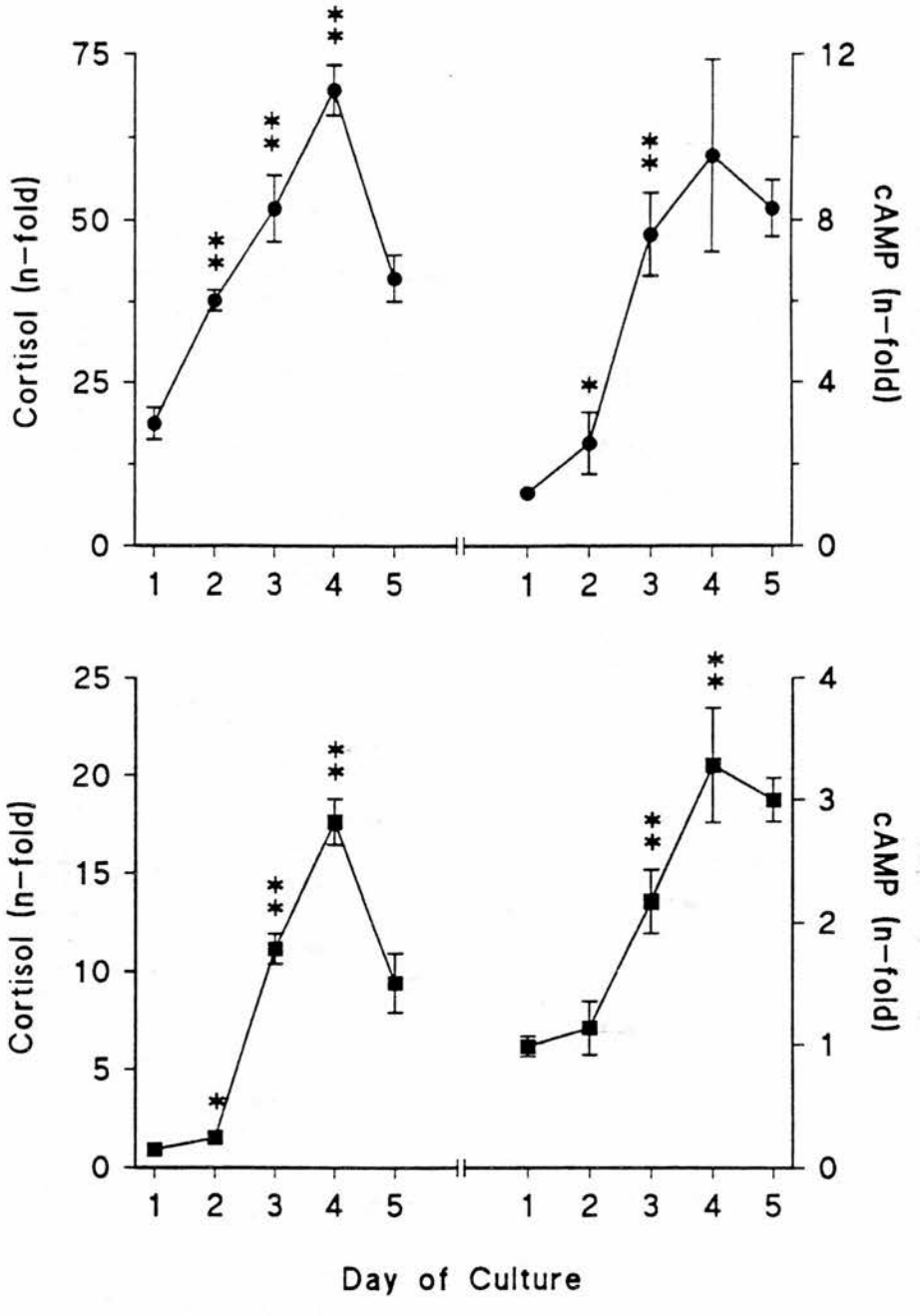

FIGURE 3.4 : Cortisol and cAMP secretory responses of bovine zfr cells stimulated for 1 h with the (Top panel) ACTH (1-24) (0.1 nM) or (Bottom panel) adrenaline (1 μM) on day of isolation (day 1) and on successive days after maintenance in primary culture (days 2-5). Results are expressed as n-fold stimulation ratios and show the mean ± s.e.m. of triplicate determinations from 3 separate cell preparations. Mean basal values for cortisol secretion were 6.80, 7.84, 13.10, 11.76 and 15.12 pmol/h/100 μg protein for days 1 to 5 respectively, and mean basal cAMP secretion was 0.129, 0.234, 0.288, 0.176 and 0.190 pmol/h/100 μg protein respectively. Results which differ significantly from the value of the previous day are indicated (*$p<0.05$, **$p<0.01$).

As previously reported (Walker *et al.* 1988), freshly isolated cells did not secrete cortisol in response to adrenaline (Figure 3.4, lower panel). However, a small but significant ($p<0.05$) response was observed following 24 h in primary culture. This response was progressively increased on days 3 and 4 before declining on day 5. The increased steroidogenic responsiveness to adrenaline was paralleled by increased cAMP formation over days 1 to 4 respectively.

3.2.5 Steroidogenic responsiveness to 8-Bromo cAMP, phorbol ester and Ca^{2+} ionophore

While steroidogenic responsiveness to ACTH and adrenaline was increased on day 2, that of AII and ACh was clearly reduced. Moreover, the loss of response to AII and ACh occurred at a time when second messenger ([^{3}H]phosphoinositol) formation in response to these agents was increased. Since different second messenger responses are associated with the actions of ACTH and adrenaline (adenylate cyclase) compared with AII and ACh (phospholipase C), it was decided to investigate the day-by day responses to 8-BrcAMP, a cell permeable analogue of cAMP, and a combination of A23187 and PMA, which mimic the effects of phospholipase C agonists by increasing $[Ca^{2+}]_i$ and stimulating protein kinase C (section 1.3.11).

Cortisol secretion in response to 8-BrcAMP increased progressively over days 1 to 4, before declining on day 5 (Figure 3.5, upper panel). In contrast, cortisol secretion in response to a combination of A23187 and PMA was reduced on day 2 ($p<0.01$) before recovering to reach a maximum on day 3, and declining thereafter (Figure 3.5, lower panel). The effects of A23187 and PMA in isolation were also studied. Although A23187 alone was relatively ineffective in stimulating cortisol secretion, no significant reduction in the cortisol response to this agent occurred on day 2. However, cortisol secretion in response to PMA alone was markedly reduced on day 2 compared with the response observed in freshly isolated cells.

3.2.6 Day-by-day changes in AII receptor number and affinity

AII receptor number and affinity were estimated on each experimental day by measuring the inhibition of ^{125}I-AII binding to cells by unlabelled AII. The binding of ^{125}I-AII reached equilibrium after 60 min at 22°C, and non-specific binding (defined as ^{125}I-AII binding in the presence of 10 μM AII) represented 1.8% of the total binding (Figure 3.6). Binding inhibition curves obtained on culture days 1-4 are shown in Figure 3.7, and the changes in AII receptor number and affinity summarised in Table 3.1. While no

FIGURE 3.5

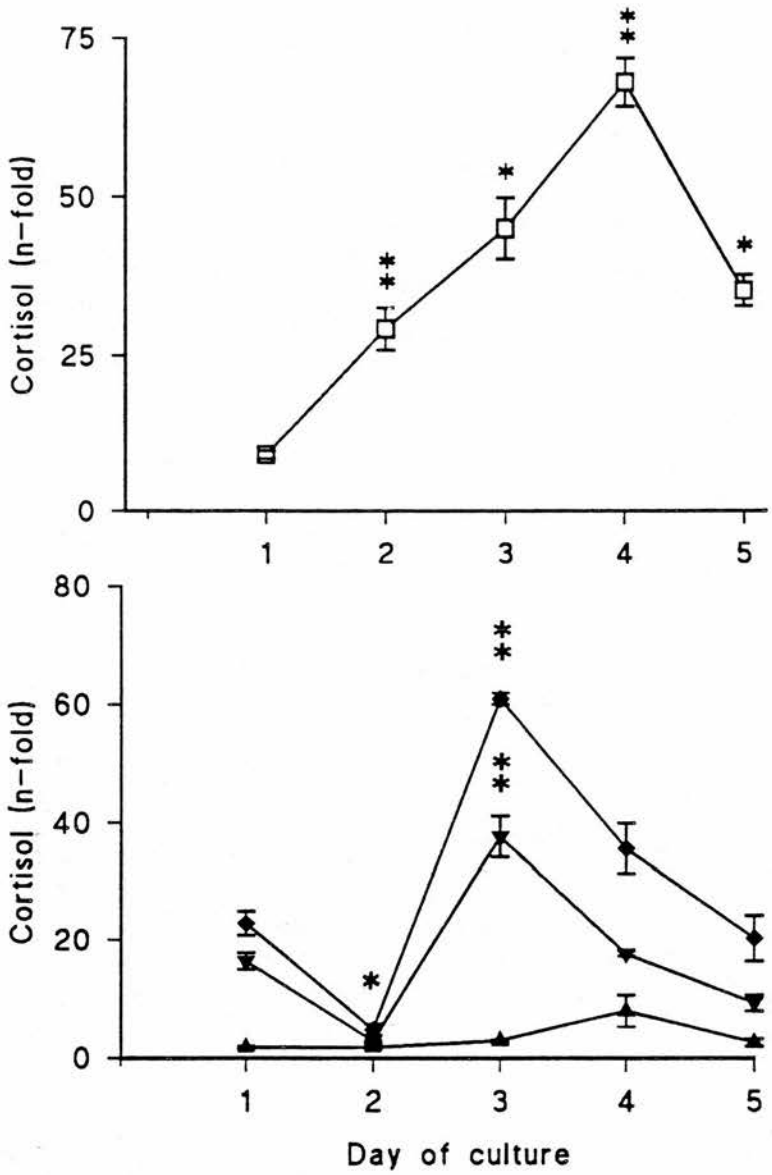

FIGURE 3.5 : (Upper panel) Cortisol response of bovine zfr cells to 8-bromoadenosine 3':5'-cyclic monophosphate (1 mM) on each of the first 5 days of primary culture. Data points are the mean ± s.e.m. of triplicate determinations from 3 separate experiments. Mean basal secretion of cortisol on days 1 to 5 was 6.80, 7.84, 13.10, 11.76 and 15.12 pmol/h/100 µg protein respectively. Values significantly different from those of the preceeding day are indicated at *$p<0.05$ and **$p<0.01$.

(Lower panel) Cortisol secretory response at A23187 alone (▲), phorbol 12-myristate 13-acetate (PMA) alone (▼) or A23187 and PMA combined (◆) (both 1 µM) on days 1 to 5 of culture. Values are the mean ± SD of triplicate determinations from a single representative experiment. Qualitatively similar results was obtained in a total of five experiments for A23187 and PMA combined, and in two experiments with A23187 and PMA alone.

FIGURE 3.6

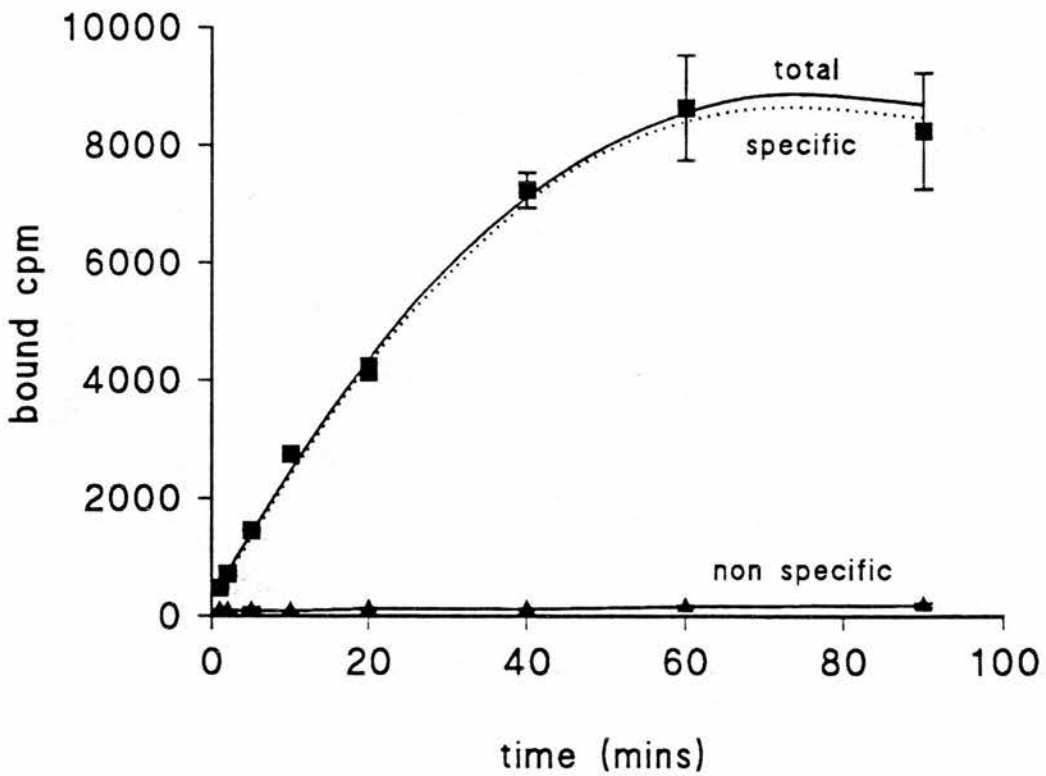

FIGURE 3.6 : Time course of ^{125}I-AII binding to day 3 primary cultures of bovine adrenal zfr cells. Cells were incubated with ^{125}I-AII (10^5 cpm, approx 0.15 nM) for the times indicated in a total volume of 1 ml EBS, at 22°C. Non-specific binding was measured in the presence of 10 µM unlabelled AII, and was 1.8% of the total bound radioactivity at 60 min.

FIGURE 3.7

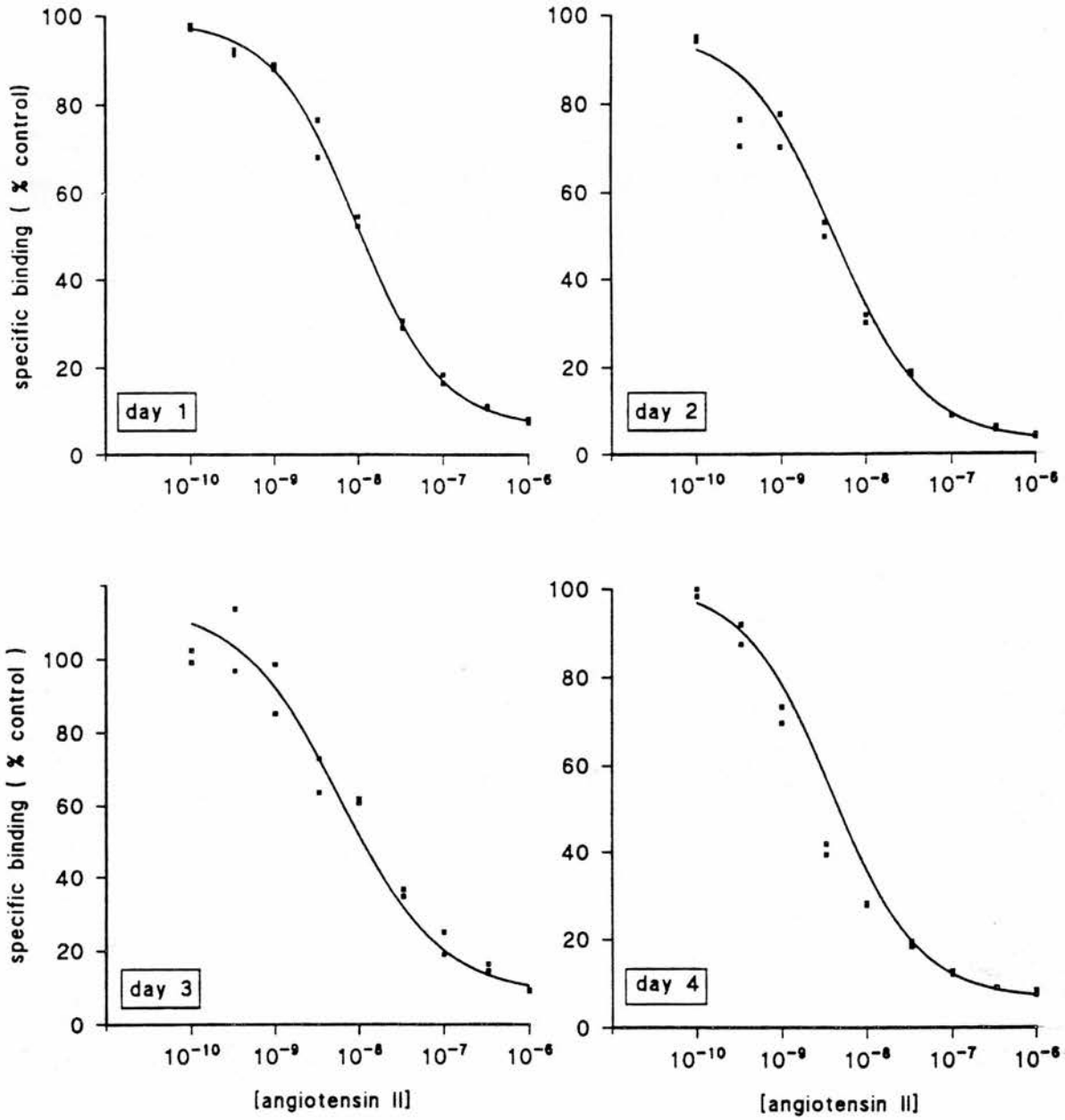

FIGURE 3.7 : Representative experiment showing inhibition of ^{125}I-AII binding to primary cultures of bovine zfr cells by unlabelled AII on each of the first 4 days of culture. Cells were incubated with ^{125}I-AII (10^5 cpm, approx. 0.15nM) for 60 min at 22°C, in a total volume of 1 ml EBS. Binding at each concentration of AII was assayed in duplicate.

TABLE 3.1

Day of culture	K_d *(nM)*	B_{max} *(fmol/mg)*
1	1.07 ± 0.21	1203 ± 408
2	2.77 ± 0.82	2661 ± 292*
3	2.62 ± 0.74	3491 ± 670
4	2.05 ± 0.53	2942 ± 687

TABLE 3.1 : Binding parameters for displacement of ^{125}I-angiotensin II by angiotensin II on each of the first four days in primary culture. Data are the mean ± s.e.m. of determinations from three separate cell preparations. Values significantly different from those of the previous day are indicated at *$p<0.05$.

difference in AII receptor affinity was detected over culture days 1-4, receptor number was significantly increased over days 1 to 3, consistant with the enhanced [^{3}H]phosphoinositol response to AII observed over this period.

3.2.7 Intracellular Ca^{2+} response to AII

Stimulation of bovine zfr cells by AII and ACh is associated with a rise in intracellular calcium concentration in response to Ins(1,4,5)P_3 produced by the action of PLC (Bird *et al*, 1990b). Figure 3.8 shows the increase in intracellular calcium in response to AII (0.1 μM) in a single representative day 2 cell following loading with the fluorescent calcium indicator fura-2. Forty cells from 5 different cell preparations were analysed in this way. An increase in intracellular calcium was observed in 68% of cells in response to AII. These findings are comparable with the intracellular calcium response to AII observed on days 3 and 4 (see chapter 7).

FIGURE 3.8

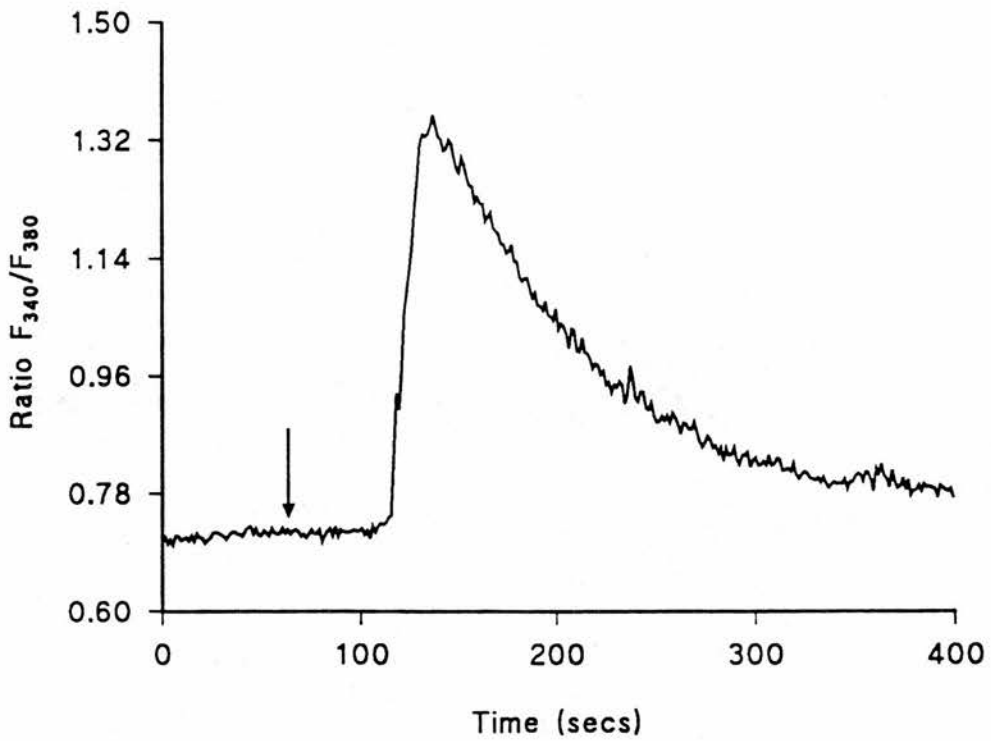

FIGURE 3.8 : Intracellular calcium response to AII (0.1 μM) from a single fura-2 loaded bovine zfr cell on day 2 of primary culture. The cell was perfused continuously with modified Kreb's buffer, and AII was infused at the time indicated (arrow). The apparent lag time of response was due to the residual 'dead volume' of the perfusion apparatus.

3.3 DISCUSSION

Consistent with previous observations (Williams *et al*, 1989; Bird *et al*, 1988; Walker *et al*, 1988), bovine zfr cells showed enhanced responsiveness to ACTH and adrenaline when maintained in primary culture, compared with the responses of freshly isolated cells. The increase in cortisol secretion in response to these agents over experimental days 1-4 was parallelled by increased formation of cAMP, possibly due to increases in receptor number or adenylate cyclase units, or indicative of a more efficient coupling of the receptor/G protein/cyclase complex. However, steroidogenic responsiveness to a constant dose of 8-BrcAMP also increased over this period, indicating that in addition to increased cAMP formation, the enhanced responses to ACTH and adrenaline may also be a result of post-second messenger factors resulting in a tighter coupling between activation of adenylate cyclase and steroidogenesis. Such factors may include an increase in the availability of cAMP-dependent protein kinases, or increased steroid synthetic capacity. Indeed, compared to freshly isolated cells, bovine zfr cells were reported to show a marked increase in cellular lipid content following primary culture (Williams *et al*, 1989). This may reflect an enhanced store of steroid precursors, and could account for the increased steroidogenic responsiveness to adenylate cyclase agonists observed in this study.

In contrast, the cortisol secretory responses to AII and ACh were reduced by 24 h in culture, and then rose to reach peak responsiveness at 48 h, before again declining. This reduced responsiveness on day 2 was not due to a reduction in cell number, as determined both by well protein content and digitonin-releasable LDH activity (Figure 3.2). Neither was the loss of the cortisol response to AII and ACh a consequence of cells plating down onto culture plastics, since qualitatively similar results were obtained when cells were maintained in suspension culture. Surprisingly, the fall in cortisol secretion on day 2 was accompanied by an increase in PLC activity in response to AII and ACh, as determined by the agonist-stimulated production of [^{3}H]phosphoinositols. Thus, the loss of responsiveness to AII and ACh was not due to reduced second messenger formation, thereby excluding reduced receptor number or affinity, G-protein availability or PLC activity as an explanation. Indeed, AII receptor number increased in parallel with [^{3}H]phosphoinositol formation in response to AII over days 1-3, and no change in AII receptor affinity was observed.

The loss of response to AII and ACh on day 2 of culture was clearly not due to a defect in the steroid synthetic or secretory mechanisms, as no reduction in sensitivity to ACTH or adrenaline was observed at this time. Indeed, steroid (cortisol) secretion increased on day

2 in response to ACTH and adrenaline. Neither was it due to a failure of the second messengers formed in response to PIC activation to elevate intracellular Ca^{2+} concentration, since a clear rise in $[Ca^{2+}]_i$ was observed in response to AII in fura-2 loaded day 2 cells. Moreover, administration of a combination of A23187 and PMA, which elevate $[Ca^{2+}]_i$ and stimulate protein kinase C independently of the receptor/ G protein complex, failed to restore the reduced cortisol response on day 2.

It is more likely that day 2 cells are unable to couple the increased Ca^{2+}/DAG signals formed on receptor activation to the activation of cytochrome P450 side-chain cleavage enzyme (P450scc), the rate limiting step of the steroid pathway (Stone & Hechter, 1955). Since the steroidogenic effect of ACTH in bovine adrenocortical cells is Ca^{2+} dependent (Yanagibashi, 1979; Kojima *et al,* 1985c), and the cortisol response to ACTH was clearly elevated on day 2, it is unlikely that the coupling of the intracellular Ca^{2+} signal to the activation of P450scc is impaired at this time. It is more likely that the loss of steroidogenic responsiveness to AII and ACh on day 2 is due to a failure of DAG formed on receptor activation to stimulate the protein kinase C signalling system, either due to a loss of protein kinase C itself, or a loss of one or more of its substrates. In support of this hypothesis, the steroidogenic effect of PMA, which mimics the effect of endogenous DAG, was markedly reduced on day 2, while that of A23187, which increases intracellular Ca^{2+} concentration, was unchanged (Figure 3.5).

These findings are of interest when compared to the ability of phospholipase C agonists to stimulate steroidogenesis in other cultured cell systems. While vasopressin has been reported to stimulate corticosterone secretion from both the pefused rat adrenal gland and from isolated superfused rat adrenocortical zfr cells (Hinson *et al,* 1987), it is not steroidogenic in 3 day primary cultures of rat zfr cells, even though stimulation of PIC activity by vasopressin can be demonstrated in this system (Gallo-Payet *et al,* 1986). A similar stimulation of PIC activity without an accompanying steroidogenic response has also been reported in Y-1 adrenal tumour cells stimulated by vasopressin or AII (Langlois *et al,* 1988,1990). Furthermore, ovine adrenocortical zf cells do not respond steroidogenically to AII, but exhibit a clear PIC response and increase in intracellular Ca^{2+} when stimulated with AII (Viard *et al,* 1990). The data presented in this chapter indicate that bovine zfr cells enter a non-responsive state similar to that of the rat and ovine cell systems described above, but recover after 24 h. This raises the possibility that rat and ovine zfr cells may respond steroidogenically to vasopressin and AII following different periods of culture.

To summarise, the day-by-day steroidogenic and second messenger responsiveness of cultured bovine zfr cells has been investigated. Although freshly isolated cells responded to ACh and AII, cells were maximally responsive to each agonist on days 3 and 4 with respect to both cortisol secretion and PIC activation. Subsequent experiments were therefore performed following 48-72 h of primary culture.

CHAPTER 4

THE MUSCARINIC RECEPTOR SUBTYPE MEDIATING ACETYLCHOLINE INDUCED CORTISOL SECRETION FROM BOVINE ZONA FASCICULATA/RETICULARIS CELLS

4.1 INTRODUCTION

Since stimulation of adrenocortical steroidogenesis in response to ACh was first demonstrated (Rosenfield, 1955), a number of studies have attempted to classify the adrenocortical ACh receptor. With the exception of the work of Rubin and Warner (1975), in which a nicotinic response to ACh was demonstrated in the feline adrenal cortex, the remaining reports over the last ten years have described a muscarinic response to ACh in both zg and zfr cells from various species (see section 1.5.5). However, the muscarinic receptor subtype(s) present in the adrenal cortex have not been identified, either by radioligand binding or functional analysis, in any species.

Muscarinic receptors are classified according to the differential affinities of selective antagonists for the various receptor subtypes (section 1.5.2). Antagonist affinity is expressed as the antagonist equilibrium dissociation constant (K_D), the ratio of association and dissociation rate constants for the combination of antagonist and receptor. Being a chemical term, K_D is largely independent of species or receptor function. The antagonist K_D can be estimated either by direct measurement of the binding of radiolabelled ligands to receptor preparations (Hulme & Birdsall, 1992), or by analysis of the antagonism of the functional response to a receptor agonist. Of the various methods of calculating K_D from functional data, two have attracted widespread use. The first method involves the inhibition of a response to a constant fixed dose of agonist by increasing concentrations of antagonist. K_D is then calculated form the inhibition curve according to the Cheng-Prusoff relationship (Cheng & Prusoff, 1973). (At present, some doubt exists regarding the correct transformation of the Cheng-Prusoff equation from enzyme kinetics to pharmacology - see Craig, 1993; Leff & Dougall, 1993). A more reliable estimate of K_D can be obtained by examining the rightward shifts of a full agonist dose-response curve in the presence of increasing concentrations of antagonist. The

degree of shift of the dose-response curve (dose ratio, r) is dependent only on the antagonist concentration [B] and its dissociation constant (K_D), which can be calculated from the Gaddum-Schild equation (Gaddum, 1937; Arunlakshana & Schild, 1959):

$$(r-1) = [B] / K_D$$

The latter method offers a number of advantages over 'Cheng-Prusoff' analysis . Firstly, although both methods are valid only for competitive antagonists, Schild analysis contains a number of criteria which must be met if the antagonism is truly competitive: a competitive antagonist should produce parallel shifts of the dose-response curve without depressing the upper asymptote. Furthermore, a plot of $\log_{10}$ (r-1) versus $\log_{10}$ [B] should be linear, with gradient not significantly different from unity, for competitive antagonism. With 'Cheng-Prusoff' analysis, the antagonism is assumed to be competitive, without any experimental justification. Secondly, the calculation of K_D from inhibition curves provides only a single estimate of K_D, while the logarithmic Schild plot described above provides one estimate for each antagonist concentration used to construct the plot: the Schild regression crosses the x axis at $\log_{10} K_D$. Finally, the experimental slope and 'linearity' of the Schild regression provides valuable information regarding the equilibrium state of the system, and any receptor sub-populations which may be present (Kenakin, 1985).

In this chapter, following initial investigation of the time course and desensitisation of the ACh response, the antagonism of ACh-stimulated cortisol secretion by a number of selective muscarinic antagonists is investigated by Schild analysis, allowing the muscarinic receptor subtype mediating ACh-induced cortisol secretion to be classified for the first time in adrenocortical cells.

4.2 RESULTS

4.2.1 Time course of ACh-stimulated cortisol secretion

Incubation of zfr cells with ACh (0.1 mM) resulted in a time -dependent increase in cortisol secretion. As shown in figure 4.1, this response was linear for up to 30 minutes, after which no further increase in cortisol secretion was observed. The absolute levels of cortisol secretion stimulated in the presence of ACh were very variable between different cell preparations, even after correction for cell number (as assessed by the well protein content), making the combination of data from different cell preparations unfeasible. However, in three out of three experiments, no significant difference between the 30 min and 60 min time points was observed.

4.2.2 Desensitisation of ACh-stimulated steroidogenesis

In order to ascertain whether the observed deviation from linearity of ACh-stimulated cortisol secretion after 30 min was due to breakdown of ACh, or represented a desensitisation phenomenon, the effect of ACh preincubation on the subsequent steroidogenic response to ACh was examined. Following exposure to ACh (0.1 mM) for various lengths of time, cells were washed thoroughly and challenged with fresh ACh (0.1 mM - 0.1 mM) for 30 min. (figure 4.2). In control cells (preincubation with vehicle alone) ACh stimulated a dose-dependent increase in cortisol secretion over the concentration range 0.1 μM - 0.1 mM. In two out of three experiments the dose response curve to ACh was significantly depressed following 30 min preincubation with ACh, with the maximum response to ACh (0.1 mM) reduced by 64% and 92% respectively. In all experiments, following 60 or 90 min preincubation, cells were completely unresponsive to further challenge with ACh. As shown in figure 4.3, this loss of responsiveness was associated with a reduction in the [^{3}H]phosphoinositiol response to ACh in [^{3}H]inositol prelabelled cells.

4.3.3 Schild analysis of the antagonism of ACh-stimulated steroidogenesis by selective muscarinic antagonists

Having established the maximum incubation time possible without inducing desensitisation, a series of cortisol dose-response curves to ACh were set up in the presence of increasing concentrations of the muscarinic antagonists pirenzepine (M_1>M_2=M_3), methoctramine (M_2>M_1=M_3), HHSD (M_1=M_3>M_2) and p-FHHSD (M_3>M_1=M_2) (figures 4.4 - 4.7, upper panels). In each experiment two different

FIGURE 4.1

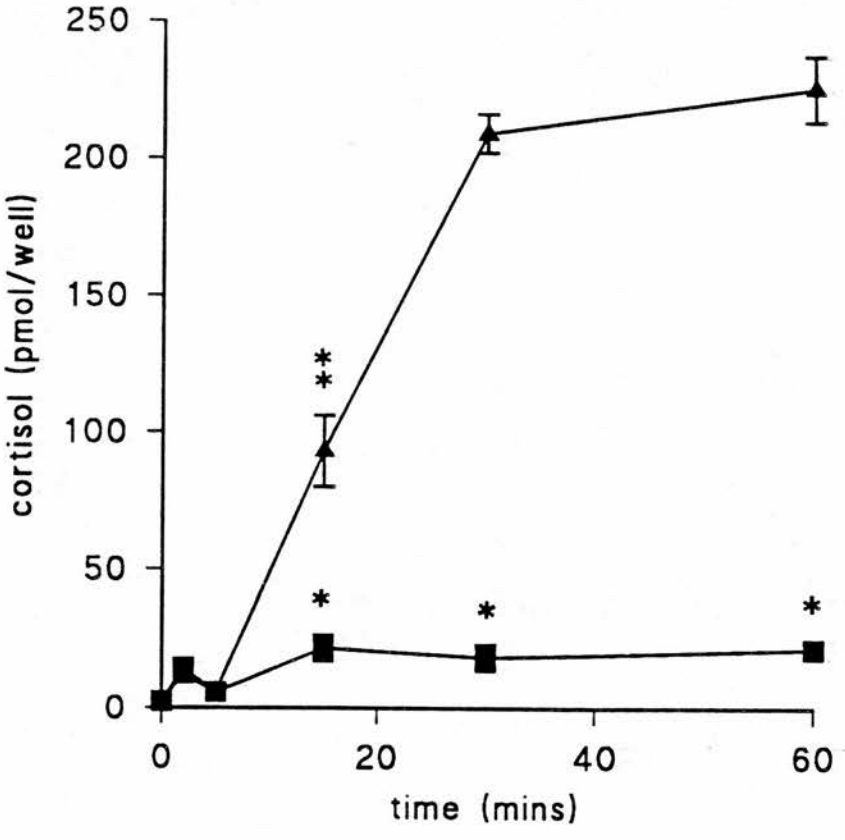

FIGURE 4.1 : Time course of ACh-stimulated cortisol secretion from bovine zfr cells. Cells were incubated with (▲) ACh (0.1 mM) or (■) vehicle for the times indicated. Data points are the mean ± SD of triplicate determinations from one representative experiment. Significant stimulation relative to time zero is shown at *p<0.05, **p<0.01.

FIGURE 4.2

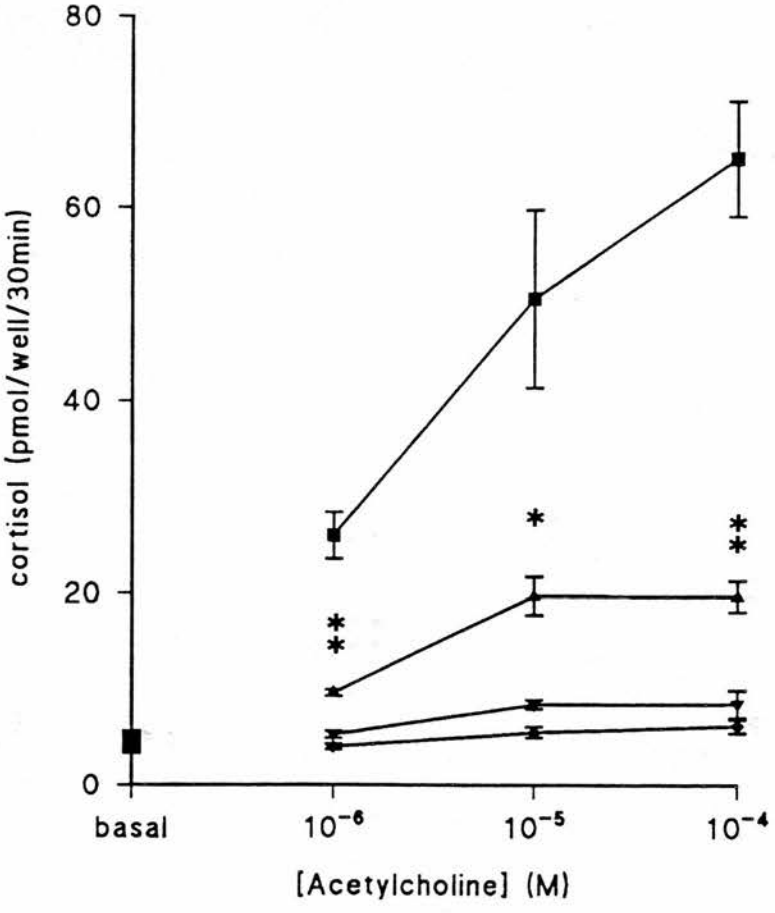

FIGURE 4.2 : Desensitisation of ACh-stimulated cortisol secretion. Cells were challenged with ACh (0.1 mM) following preincubation with ACh (0.1 mM) for the following peroids of time: (■) zero; (▲) 30 min; (▼) 60 min; (◆) 90 min. Data show the mean ± SD of triplicate determinations from a single representative experiment. Significant inhibition relative to control (no preincubation) values is indicated at *$p<0.05$, **$p<0.01$.

FIGURE 4.3

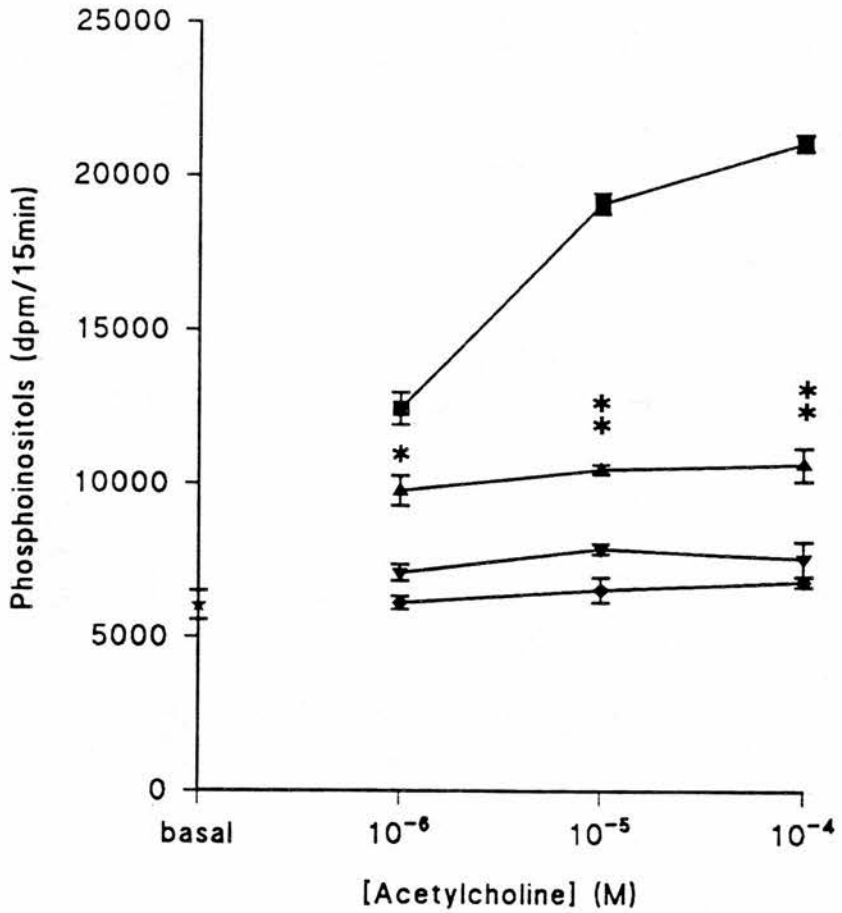

FIGURE 4.3 : Desensitisation of ACh-stimulated phosphoinositol production. Cells were challenged with ACh (0.1 mM) following preincubation with ACh (0.1 mM) for the following peroids of time: (■) zero; (▲) 30 min; (▼) 60 min; (◆) 90 min. Data show the mean ± SD of triplicate determinations from a single representative experiment. Significant inhibition relative to control (no preincubation) values is indicated at *$p<0.05$, **$p<0.01$.

concentrations of antagonist were assessed, and six such experiments from different cell preparations performed for each antagonist to generate data for Schild analysis.

Basal cortisol secretion was 10.4 ± 1.4 pmol/well/30 min (mean ± s.e.m., n = 24 experiments). The dose response curve for ACh-stimulated cortisol secretion fell in the concentration range 0.1 μM - 10 mM, and the ED_{50} for ACh was 1.1 μM (range 0.4 μM - 7.2 μM). Maximal stimulation of cortisol secretion occurred at 10 μM ACh, at which concentration cortisol secretion was 143.4 ± 12.9 pmol/well/30 min (mean ± s.e.m., n = 24).

The effect of each of the antagonists studied was to shift the log dose response curve to ACh to the right. None of the antagonists had any effect on the maximal response attainable by ACh, or on the slope of the dose response curve (figures 4.3 - 4.7, upper panels). The rank order of antagonist potency was HHSD > p-FHHSD > pirenzepine > methoctramine. For estimation of antagonist affinities, the dose-ratio (r) for each concentration of antagonist was obtained and Schild plots ($\log_{10}$ (r-1) versus $\log_{10}$ [antagonist]) constructed (figures 4.4 - 4.7, lower panels). The estimated pA_2 values and Schild slopes for each antagonist are shown in table 4.1.

FIGURE 4.4

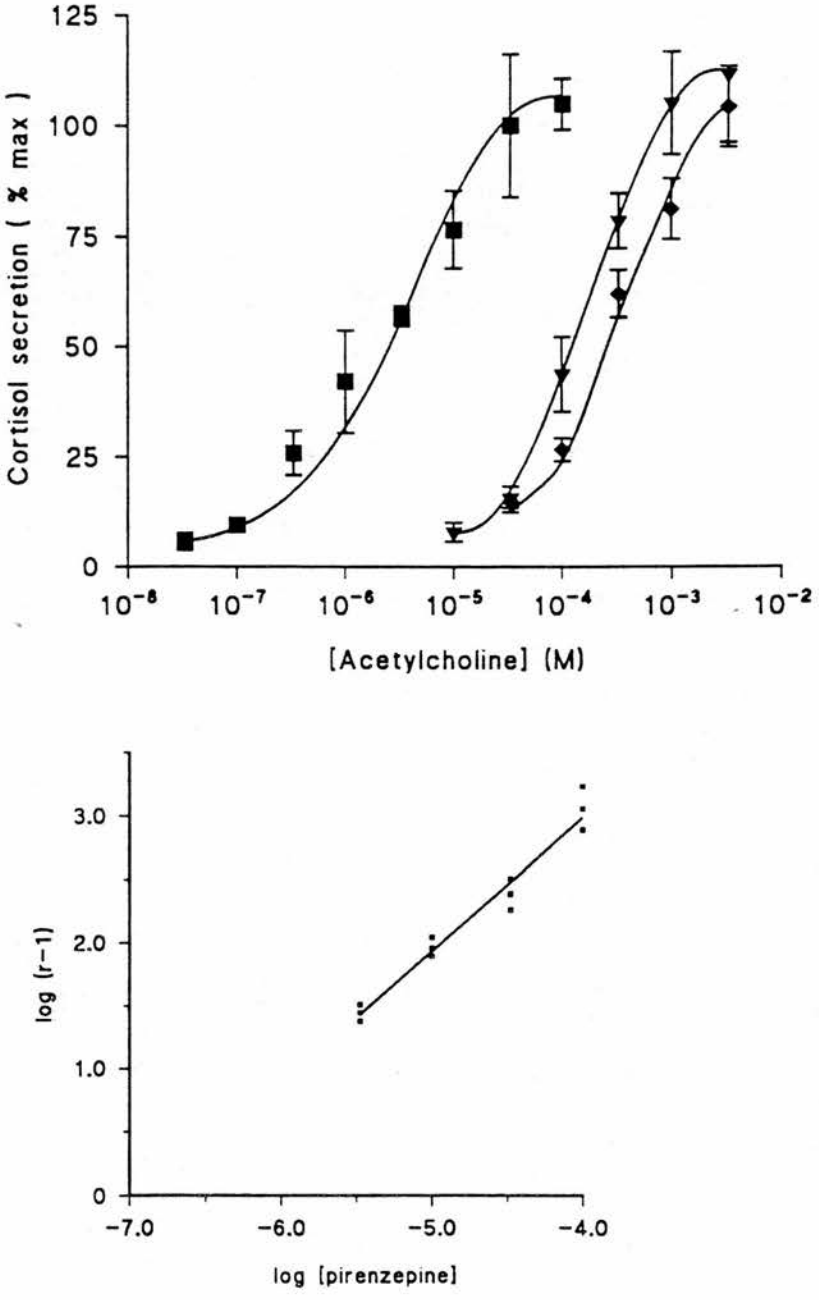

FIGURE 4.4 : (Upper panel) Concentration response curves for the secretion of cortisol produced on stimulation with ACh alone (■) and in the presence of pirenzepine (▼) 3.3μM, (◆) 10μM. Data points are the mean ± SD of triplicate values from a representative experiment.

(Lower panel) Schild regression of the antagonism of ACh-stimulated cortisol secretion by pirenzepine. Cumulative data from 6 experiments from separate cell preparations (2 concentrations of antagonist per experiment).

FIGURE 4.5

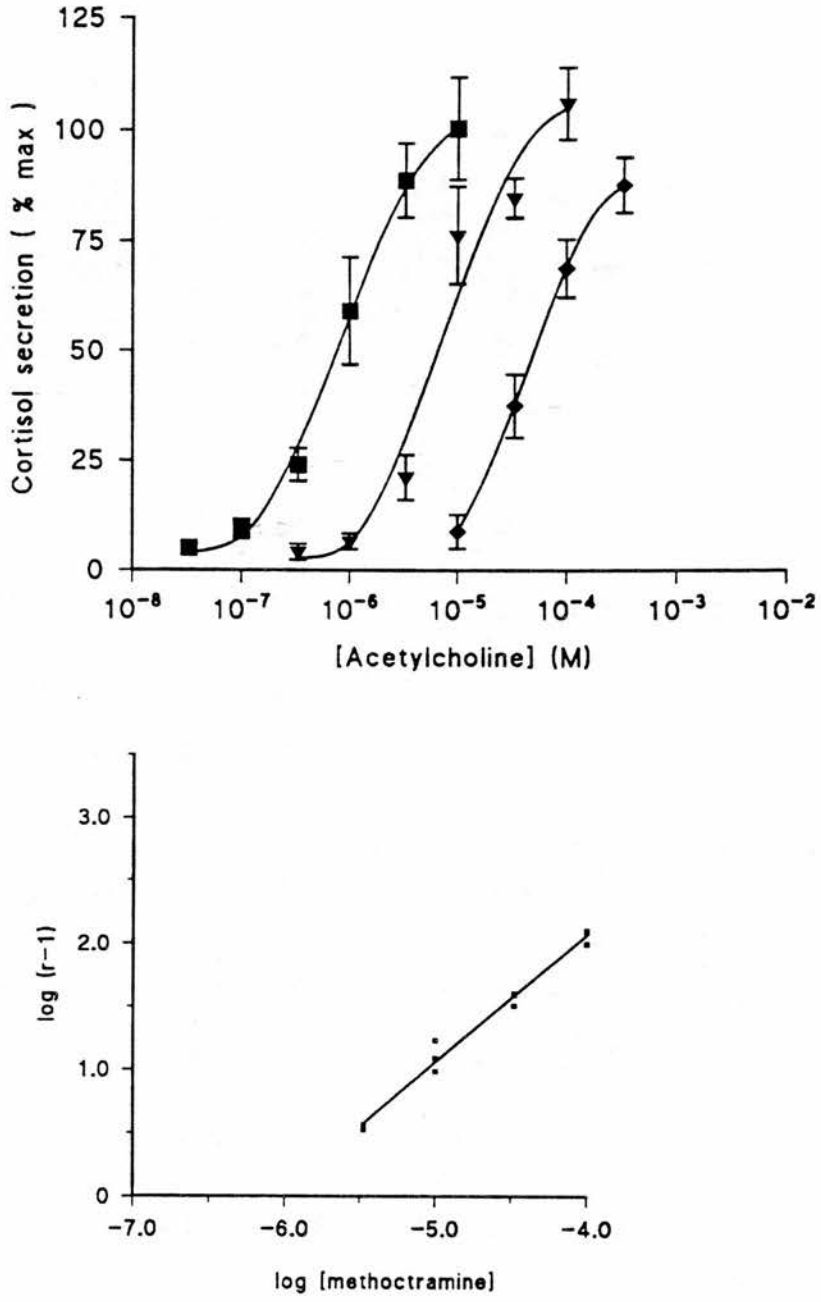

FIGURE 4.5 : (Upper panel) Concentration response curves for the secretion of cortisol produced on stimulation with ACh alone (■) and in the presence of methoctramine (▼) 10 μM, (◆) 100 μM. Data points are the mean ± SD of triplicate values from a representative experiment.

(Lower panel) Schild regression of the antagonism of ACh-stimulated cortisol secretion by methoctramine. Cumulative data from 6 experiments from separate cell preparations (2 concentrations of antagonist per experiment).

FIGURE 4.6

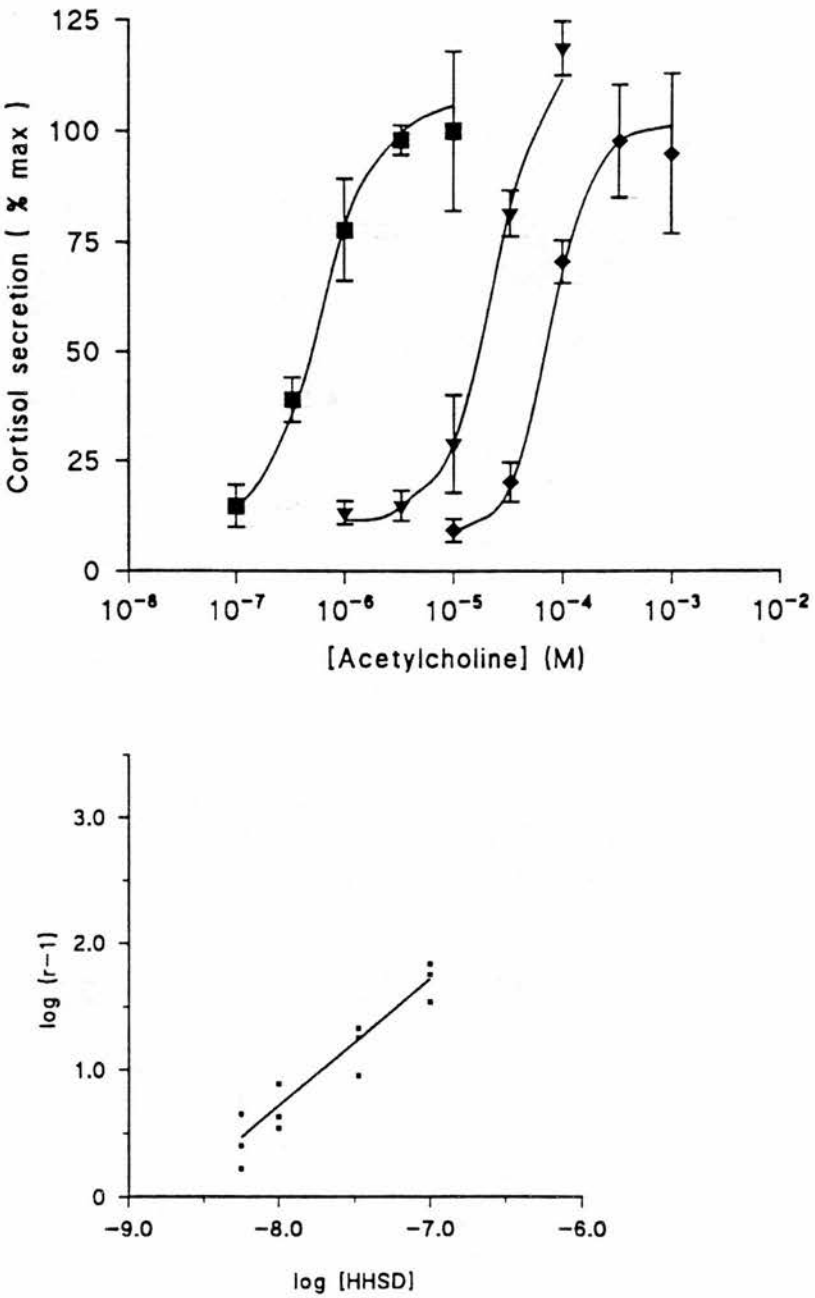

FIGURE 4.6 : (Upper panel) Concentration response curves for the secretion of cortisol produced on stimulation with ACh alone (■) and in the presence of HHSD (▼) 33 nM, (◆) 100 nM. Data points are the mean ± SD of triplicate values from a representative experiment.

(Lower panel) Schild regression of the antagonism of ACh-stimulated cortisol secretion by HHSD. Cumulative data from 6 experiments from separate cell preparations (2 concentrations of antagonist per experiment).

FIGURE 4.7

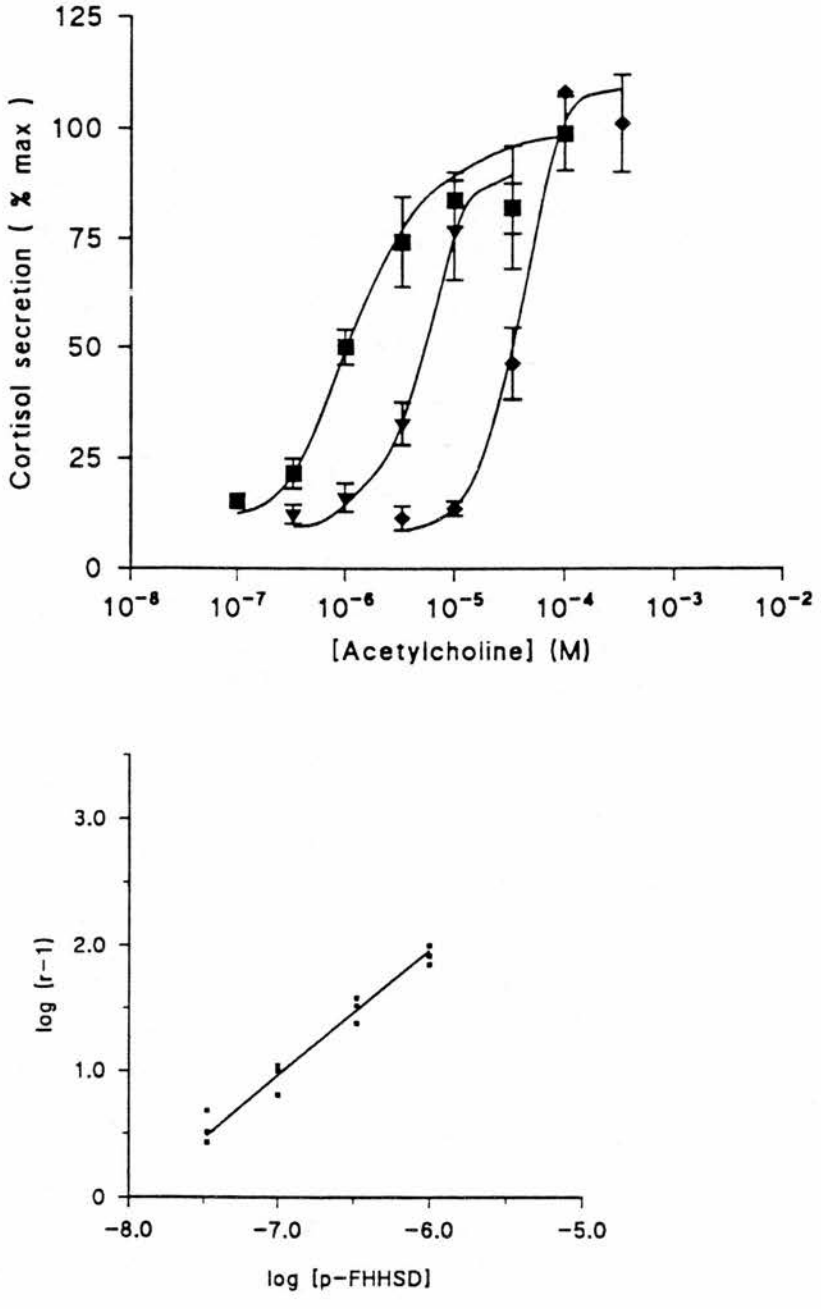

FIGURE 4.7 : (Upper panel) Concentration response curves for the secretion of cortisol produced on stimulation with ACh alone (■) and in the presence of p-FHHSD (▼) 33 nM, (◆) 333 nM. Data points are the mean ± SD of triplicate values from a representative experiment.

(Lower panel) Schild regression of the antagonism of ACh-stimulated cortisol secretion by p-FHHSD. Cumulative data from 6 experiments from separate cell preparations (2 concentrations of antagonist per experiment).

TABLE 4.1

Antagonist	*pA2 ± s.e.m.*	*slope ± s.e.m.*	*Published pA2***		
			M_1	M_2	M_3
pizenzepine	6.95 ± 0.28	1.06 ± 0.06†	8.24	6.82	6.88
methoctramine	6.06 ± 0.27	1.01 ± 0.04†	6.85	7.69	6.17
HHSD	8.68 ± 0.28	1.02 ± 0.10†	7.92	6.53	7.96
p-FHHSD	7.96 ± 0.29	0.95 ± 0.05†	6.68	6.01	7.84

†Slope not significantly different from unity

**Data from Lambrecht *et al* (1989)

TABLE 4.1 : Comparison of experimental pA_2 values for pirenzepine, methoctramine, HHSD and p-FHHSD in zfr cells with previously published values.

4.3 DISCUSSION

Bovine adrenocortical ACh receptors have previously been characterised as muscarinic both in the outer zg and the inner zfr (Kojima *et al,* 1986a; Hadjian *et al,* 1982). Walker *et al* (1990) reported the effects of a number of cholinergic agonists and antagonists on cortisol secretion from bovine zfr cells, obtaining preliminary evidence for the presence of the M_3 receptor subtype. However, definitive classification of the muscarinic receptor subtypes present in the adrenal cortex has not been carried out in any species, either by ligand binding or functional analysis. The data presented in this chapter establish that ACh-stimulated cortisol secretion in bovine zfr cells occurs through activation of the M_3 receptor, as discussed below.

Preliminary experiments designed to estimate antagonist affinity in zfr cells were conducted with an incubation time of 60 min, and produced inconsistent and irreproducible results (data not shown). Investigation of the time-course of ACh-stimulated cortisol secretion revealed a deviation from linearity after 30 min stimulation (figure 4.1), indicating that quantitative measurements made after this time were not made at equilibrium. It seemed unlikely that the observed plateau at 30 min was due to breakdown of ACh: although the concentration of cholinesterase present in the culture system was not assayed, experiments were performed in a simple balanced salts solution after extensive washing of the cells. This would be expected to completely remove any residual cholinesterase activity which may have been present in the complete culture medium. As shown in figure 4.2, the observed deviation from linearity was due to a rapid desensitisation of zfr cells to the agonist. Desensitisation in response to ACh is well documented in other tissues (Higuchi *et al,* 1985), although there is little information on the phenomenon in adrenocortical cells. Superfused frog interrenal cells stimulated with ACh exhibited a marked desensitisation with respect to both corticosterone and aldosterone secretion following 20 min exposure to ACh (Benyamina *et al,* 1987). The time course of desensitisation in these two adrenocortical preparations therefore appears similar. As reported for muscarinic receptor desensitisation in other systems (Kopp *et al,* 1990), the loss of functional response to ACh was accompanied by a progressive reduction in second messenger (phosphoinositol) production following agonist preincubation. This could result from either internalisation and inactivation of agonist bound receptors, or by the protein kinase C-mediated inhibition of phospholipase C (Orenella *et al,* 1985; Nishizuka, 1956; Liles *et al,* 1986; L'Alleman *et al,* 1986).

Having established conditions for stimulation which did not cause desensitisation, the effect of selective muscarinic antagonist on ACh-stimulated cortisol secretion was

assessed. Each of the antagonists employed produced parallel shifts of the log dose response curve to ACh without depressing the maximum response to ACh, and produced linear Schild regressions with slopes not significantly different from unity, consistent with competitive antagonism (Kenakin, 1987). The rank potency order (HHSD > p-FHHSD > pirenzepine > methoctramine) is characteristic of that of the M_3 receptor (Lambrecht *et al*, 1989). Furthermore, the experimental pA_2 values obtained agree closely with previously published values at M_3 receptors: the relatively low potencies of pirenzepine (pA_2 = 6.95) and methoctramine (pA_2 = 6.06) suggest that the steroidogenic response to ACh is not mediated by M_1 or M_2 receptors respectively (see Mei *et al* (1989) for review), while the high pA_2 values for HHSD (pA_2 = 8.68) and p-FHHSD (pA_2 = 7.96) are characteristic of M_3 receptor function (Fuder *et al*, 1985; Lambrecht *et al*, 1988).

Recently a fourth muscarinic cholinoceptor has been pharmacologically characterised (M_4) (Lazareno *et al*, 1990; Garcia-Villalon *et al*, 1991) which appears to correspond to the m4 gene product (Tietje *et al*, 1991). While the bovine adrenal medulla has been shown to express mRNA transcripts exclusively for the m4 receptor, a weak signal for m4 receptor mRNA was also detected in the cortex (Fernando *et al*, 1991). Although the M_4 subtype exhibits high affinity for the M_3 -selective compounds HHSiD and p-FHHSiD (Lazareno *et al*, 1990), it also has a moderate to high affinity for methoctramine similar to that of pirenzepine in both ligand binding and functional assays (Lazareno *et al*, 1990; Caulfield & Brown, 1991). The low pA_2 for methoctramine observed in bovine zfr cells argues against any significant involvement of the M_4 receptor in ACh-stimulated cortisol secretion, although the existence of this receptor subtype in zfr cells cannot be excluded on the basis of the present data.

While the observed pA_2 values for pirenzepine, methoctramine and p-FHHSD correlated well with the known values at M_3 receptors in other systems (Mei *et al* (1989), and Table 1) that for HHSD was slightly higher than would be expected to occur at any muscarinic receptor subtype. While this result is not inconsistent with an M_3 receptor classification, since HHSD exhibits highest affinity at M_1/M_3 receptors, it does require comment. It is possible that this reflects a species-specific heterogeneity between M_3 receptors arising from differences in receptor structure or G protein coupling. A similarly high affinity for HHSD (pA_2 = 8.5) has been reported previously at M_3 receptors in ovine detrussor smooth muscle (Rivera *et al*, 1991). Although their study used carbachol as the agonist, this would not be expected to affect the apparent antagonist affinity, since measurements of antagonist pA_2 values by Schild analysis are independent of the agonist used to elicit the functional response. The possibility that the unusually high pA_2 value for HHSD

obtained in the present study reflects an error in the assay method was also considered. HHSD is known to be a "sticky" compound, and it is feasible that drug adsorption onto experimental plastics caused errors in the dilution of the antagonist. This seems unlikely however, since an incomplete solution of the antagonist would be expected to result in a lower, rather that a higher apparent pA_2 value. Also unlikely is the possibility that inadequate antagonist equilibration time was allowed before measurement of dose ratios. The existence of a non-equilibrium steady state would be expected to produce a Schild regression of slope less than unity (Kenakin, 1985,1987): the experimental Schild slopes for each antagonist were not significantly different from unity.

The presence of the M_3 muscarinic receptor is consistent with the previously reported effects of ACh in bovine zfr cells : (i) stimulation of phospholipase C activity (Hadjian *et al,* 1984a; Walker *et al,* 1990), the second messenger system associated with the cloned m1, m3 and m5 gene products, and with native M_1 and M_3 cholinoceptors (Bonner, 1989); (ii) the lack of effect of ACh on cAMP levels in adrenocortical cells (Hadjian *et al,* 1982; Walker *et al,* 1990); (iii) the inability of the M_1 selective agonist McN-A-343 to stimulate cortisol secretion from bovine zfr cells (Walker *et al,* 1990).

Although the significance of ACh in regulating adrenocortical steroidogenesis remains to be established, a number of observations support an *in vivo* function. ACh stimulates cortisol secretion from both the isolated perfused adrenal gland and from freshly isolated bovine zfr cells (Rosenfield, 1955; Walker *et al,* 1990). In addition, acetylcholinesterase-positive innervation of the adrenal cortex has been detected in several species, including sheep and man (Robinson *et al,* 1977; Charlton *et al,* 1991), and a possible role for ACh in regulating steroidogenesis in man has been proposed (Stern *et al,* 1989).

In conclusion, the antagonist affinity profile presented in this chapter most closely resemble that of the M_3 receptor, and it is proposed that this receptor subtype mediates ACh-stimulated cortisol secretion from bovine zfr cells.

CHAPTER 5

THE RECEPTOR SUBTYPE MEDIATING ANGIOTENSIN II INDUCED CORTISOL SECRETION FROM BOVINE ZONA FASCICULATA/RETICULARIS CELLS

5.1 INTRODUCTION

The development of non-peptide antagonists of AII has allowed the unambiguous demonstration of two subtypes of AII receptor, termed AT_1 and AT_2 (reviewed in Timmermans *et al*, 1993). The pharmacology, function and tissue distribution of these receptor subtypes, together with the evidence suggesting the existence of further subtypes of AII receptor, have been discussed in section 1.6. With regard to adrenocortical AII receptors, binding studies have identified both AT_1 and AT_2 sites in the adrenal cortex of rat, rabbit, monkey and human species (Chiu *et al,* 1989a; Whitbread *et al,* 1989; Chang & Lotti, 1991). By contrast, in the bovine adrenal cortex, the majority of AII receptors appear to belong to the AT_1 class, and AT_2 sites have been found to be either completely absent (Balla *et al,* 1991), or present at very low density (Ouali *et al,* 1992).

In bovine adrenal zg cells, the effects of AII on aldosterone secretion and inhibition of ACTH-stimulated cAMP formation are mediated by AT_1 receptors (Balla *et al,* 1991). In addition, growth of the bovine adrenocortical AC1 cell line in response to AII occurs via AT_1 receptors (Natarajan *et al,* 1992). However, the receptor subtype mediating the effects of AII in bovine zfr cells has not been characterised, and it is the aim of this chapter to characterise the receptor subtype mediating AII induced cortisol secretion from bovine zfr cells.

In view of the increasing evidence suggesting the existence of subtypes of AT_1 receptors (see section 1.6.2) it is perhaps insufficient to simply demonstrate sensitivity of a given response to a selective AII antagonist in order to make statements about the receptor mediating the response. If the current system of muscarinic receptor classification is considered, in which selective antagonists are active at all muscarinic receptor subtypes, but differ only in their degree of potency between subtypes, then the need for accurate

determination of antagonist potency at AII receptors becomes clear. Few investigators have reported pA_2 values for non-peptide AII antagonists, and no such measurements have been made in adrenocortical preparations. For these reasons, it was decided to use Schild analysis to obtain antagonist pA_2 values in bovine zfr cells.

Following a recent report that multiple AII receptors could be identified in rat tissues by isoelectric focusing (Jimenez *et al*, 1991), it was also decided to apply this methodolgy to membrane fractions prepared from bovine zfr cells. Isoelectric focusing has been used for many years as a preparative or analytical method for separating molecules, especially proteins, according to differences in their isoelectric point (p*I*), the pH at which the molecule exhibits a net charge of zero (reviewed in Rilbe, 1976). The technique depends on the properties of ampholines, polyamino/polycarboxylic acids which line up to form a natural pH gradient in the presence of an electric field due to their high conductance and buffering capacity. When incorporated into polyacrylamide gels, ampholines form a pH gradient across the gel through which proteins migrate to the region of the gel corresponding to their p*I*. The position of the proteins in the gradient can be detected by staining, enzymatic, or, in the case of radiolabelled proteins, by autoradiographic means. Importantly, the p*I* of a protein is independent of molecular size, and, since very small differences between molecules may have a large effect on their p*I*, closely related molecules can be separated by this method.

5.2 RESULTS

5.2.1 Effect of selective and non-selective antagonists on AII-stimulated cortisol secretion and PLC activity

Previous studies have established that AII-stimulated cortisol secretion from bovine zfr cells is mediated through the activation of PLC (see section 1.6.4). Cells prelabelled with [^{3}H]inositol for 48 h secrete cortisol and produce aqueous [^{3}H]phosphoinositols in response to AII with similar dose-dependency (Bird *et al*, 1989). The effects of the non-selective antagonist saralasin and the AT_1-selective antagonist losartan on AII-stimulated cortisol secretion and [^{3}H]phosphoinositol production are shown in Figures 5.1 and 5.2 respectively.

Mean basal cortisol secretion was 5.37 $\pm$ 0.83 pmol/well/h, and secretion stimulated by a maximally effective concentration of AII (10 nM) was 491.4 $\pm$ 53.1 pmol/well/h. Both saralasin and losartan dose-dependently inhibited cortisol secretion in response to AII (10 nM) with ID_{50} values of 120 nM (range 62 - 150 nM) and 3.1 μM (range 0.2 - 7.9 μM) respectively (Figure 5.1). Complete inhibition of AII-stimulated cortisol secretion occurred at 10 μM saralasin and 100 μM losartan.

Mean basal [^{3}H]phosphoinositol production was 4774 $\pm$ 286 dpm/well/15 min. In the presence of AII (10 nM) [^{3}H]phosphoinositol production was 59581 $\pm$ 8960 dpm/well/15 min. As shown in Figure 5.2, the production of [^{3}H]phosphoinositols in response to AII was also dose-dependently inhibited both by saralasin and losartan with ID_{50} values of 51 nM (range 19 - 69 nM) and 0.49 μM (range 0.3 - 1.1 μM) respectively.

Figure 5.3 shows the effect of the AT_2-selective antagonist PD123177 on AII-stimulated cortisol secretion and PLC activity. PD1231777 did not significantly affect the magnitude of the steroidogenic or second messenger responses to AII at concentrations up to 0.1 mM antagonist. However, a small but non-significant depression of AII-stimulated [^{3}H]phosphonositol secretion was observed at low (1 - 10 nM) concentrations of PD123177.

5.2.2 Effect of saralasin and losartan on the cortisol dose-response curve to AII

To further characterise the antagonism of AII-stimulated cortisol secretion by saralasin and losartan, Schild analysis was performed to obtain antagonist pA_2 values. Representative experiments are shown in Figures 5.4 and 5.5. Both saralasin (10 nM -

FIGURE 5.1

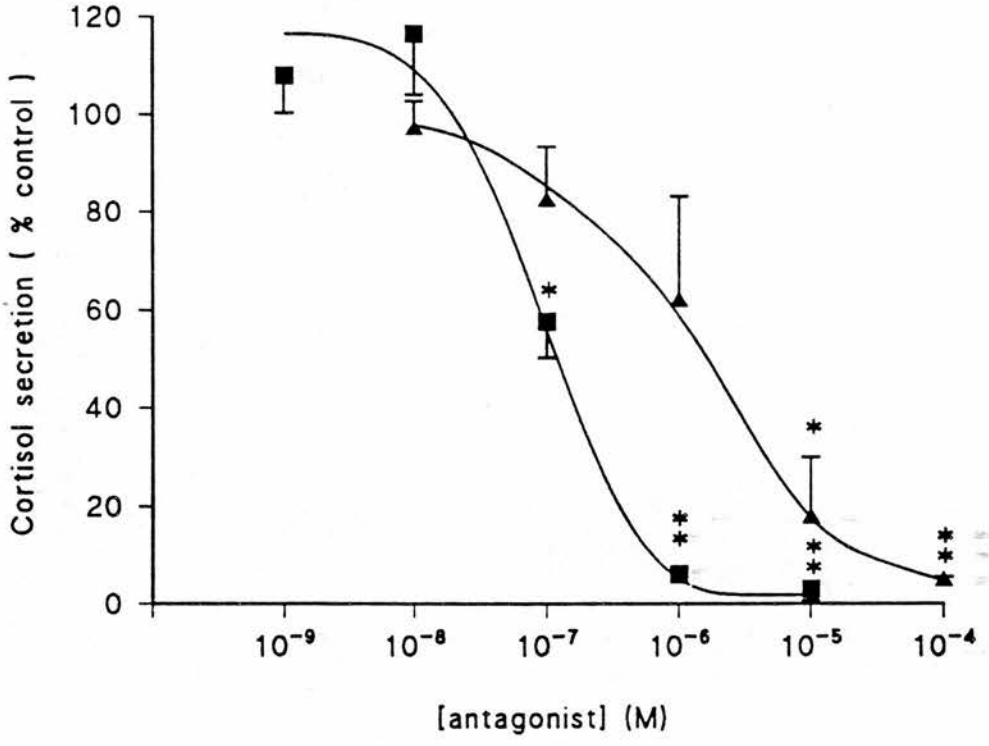

FIGURE 5.1 : Effect of saralasin and losartan on AII-stimulated cortisol secretion by bovine zfr cells. Cells were incubated with either AII (10 nM) alone, or AII (10 nM) in the presence of increasing concentrations of (■) saralasin or (▲) losartan. Values are expressed as a percentage of the control (AII, 10 nM) response, and are the mean ± s.e.m. of triplicate determinations from three independent cell preparations. Significant inhibition relative to the control AII response is indicated at (*) $p < 0.05$ and (**) $p < 0.01$.

FIGURE 5.2

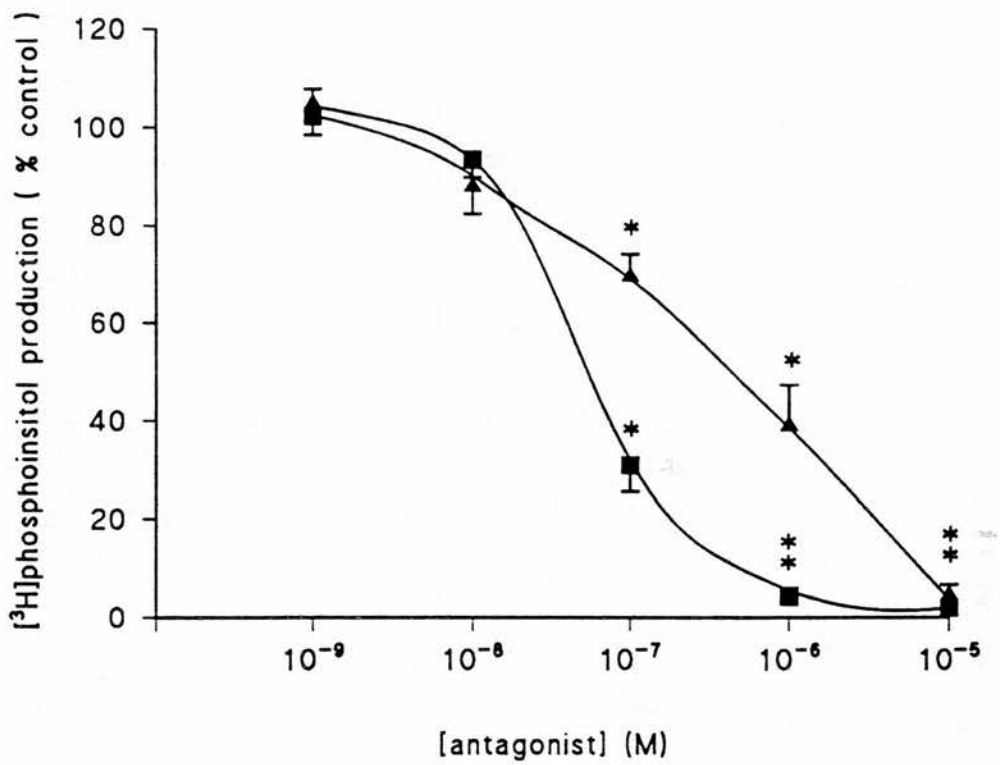

FIGURE 5.2 : Effect of saralasin and losartan on AII-stimulated phosphoinositol production by bovine zfr cells. Cells were incubated with either AII (10 nM) alone, or AII (10 nM) in the presence of increasing concentrations of (■) saralasin or (▲) losartan. Values are expressed as a percentage of the control (AII, 10 nM) response, and are the mean ± s.e.m. of triplicate determinations from three independent cell preparations. Significant inhibition relative to the control AII response is indicated at (*) $p < 0.05$ and (**) $p < 0.01$.

FIGURE 5.3

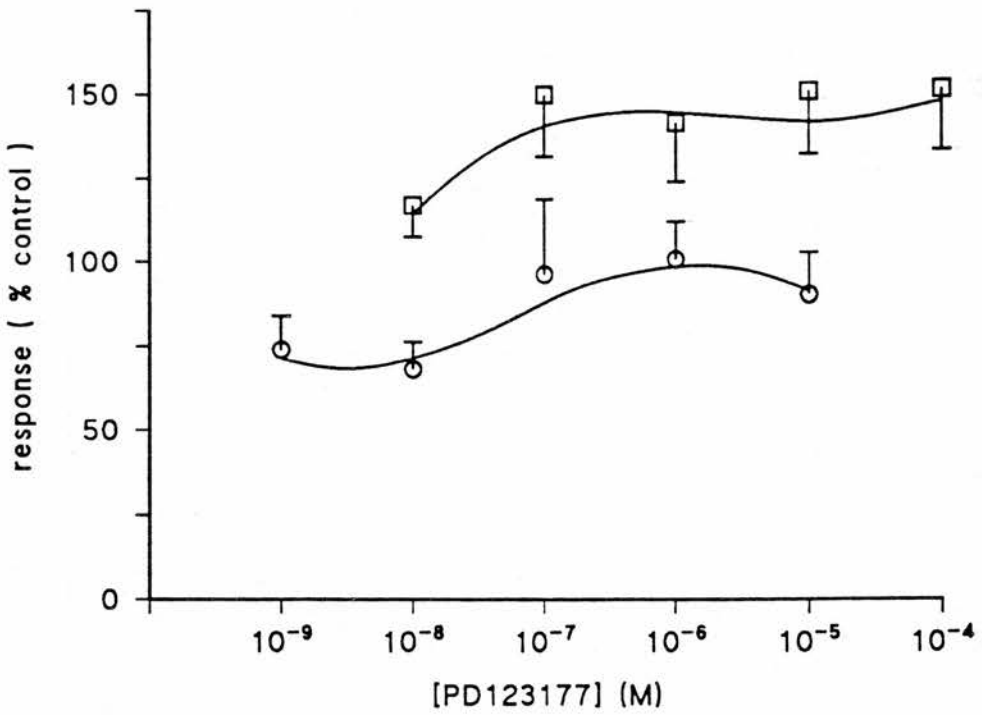

FIGURE 5.3 : Effect of PD123177 on AII-stimulated cortisol secretion (❑) and phosphoinositol production (○) by bovine zfr cells. Cells were incubated with either AII (10 nM) alone, or AII (10 nM) in the presence of increasing concentrations of PD123177. Data points are expressed as a percentage of the control (AII, 10 nM) response, and show the mean ± s.e.m. of triplicate determinations from three independent cell preparations. Significant inhibition relative to the control AII response is indicated at (*) $p < 0.05$ and (**) $p < 0.01$.

0.33 μM) and losartan (0.33 μM - 10 μM) produced parallel rightwards shifts of the dose-response curve to AII without depressing the maximum response attainable by AII, consistent with competitive antagonism. The resulting Schild regressions are shown in Figure 5.6, and the extrapolated pA_2 values and Schild slopes for saralasin and losartan, together with previously published values for these antagonists, summarised in Table 5.1.

5.2.3 Separation of AII receptor binding proteins by isoelectric focusing

Membrane fractions from bovine zfr cells were labelled with ^{125}I-AII and fractionated by isoelectric focusing on polyacrylamide gels containing pharmalyte pH 5.0 - 8.0 (Section 2.8). This procedure establishes a pH gradient across the gels (Figure 5.7), through which proteins migrate to the region of the gel corresponding to their isoelectric point (p*I*). Following autoradiography, at least two distinct ^{125}I-AII binding proteins were visible (Figure 5.8), of p*I* 7.6 and 7.3 respectively. In two out of five experiments, a third very faint band was visible at pH 7.1 (Figure 5.8). In order to identify these binding proteins, incubations were carried out in the presence of selective antagonists. PD123177 (10 μM) had no effect on the binding to either the major (p*I* 7.3) or the minor (p*I* 7.6) protein, while in the presence of losartan (10 μM) ^{125}I-AII binding to both proteins was reduced to levels similar to the non-specific binding defined in the presence of 10 μM unlabelled AII.

FIGURE 5.4

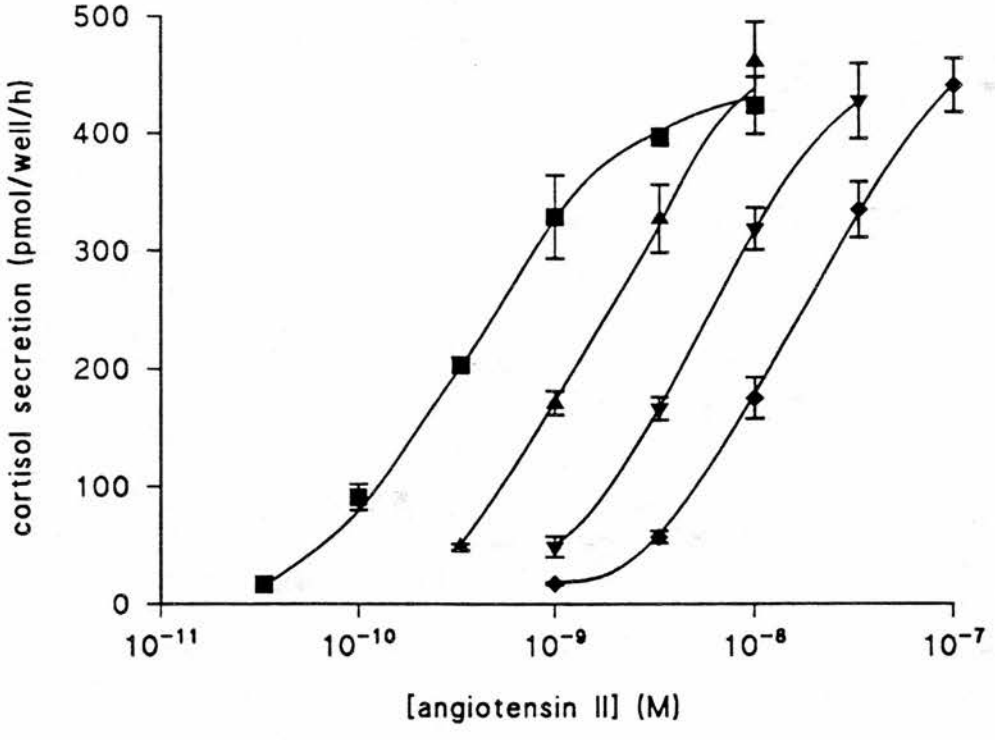

FIGURE 5.4 : Representative experiment showing the effect of saralasin on the dose response curve for AII-stimulated cortisol secretion from bovine zfr cells. Cells were incubated with AII at the concentrations indicated either in the absence of antagonist (■), or in the presence of saralasin at the following concentrations: (▲), 10 nM; (▼), 33 nM; (◆), 100 nM. Values are the mean ± SD of triplicate determinations.

FIGURE 5.5

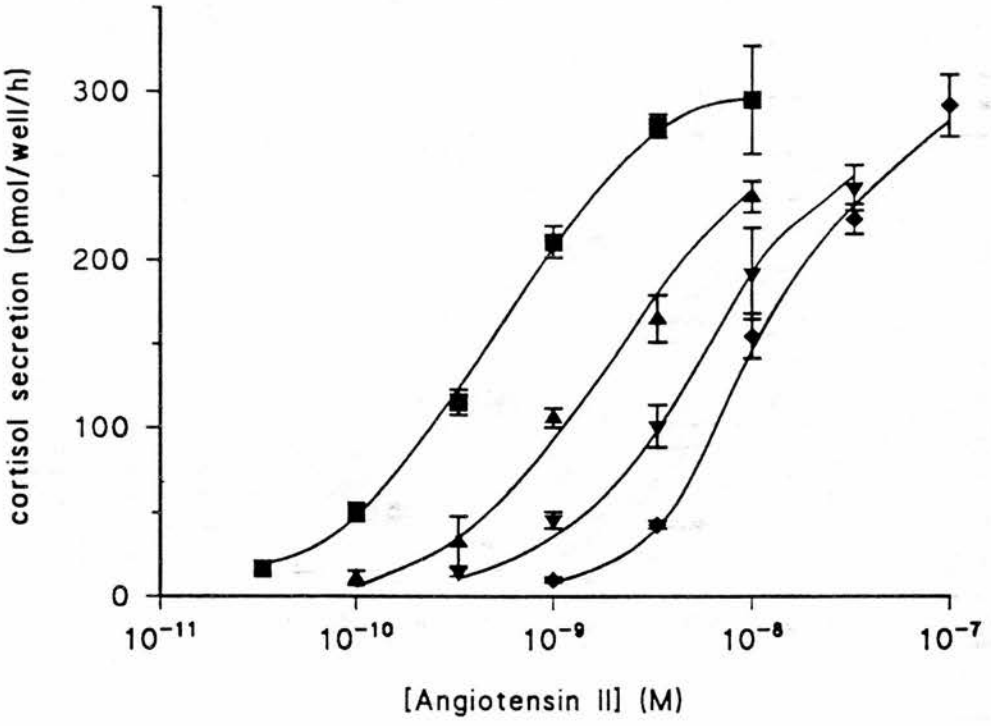

FIGURE 5.5 : Representative experiment showing the effect of losartan on the dose response curve for AII-stimulated cortisol secretion from bovine zfr cells. Cells were incubated with AII at the concentrations indicated either in the absence of antagonist (■), or in the presence of losartan at the following concentrations: (▲), 0.33 μM; (▼), 1.0 μM; (◆), 3.3 μM. Values are the mean ± SD of triplicate determinations.

FIGURE 5.6

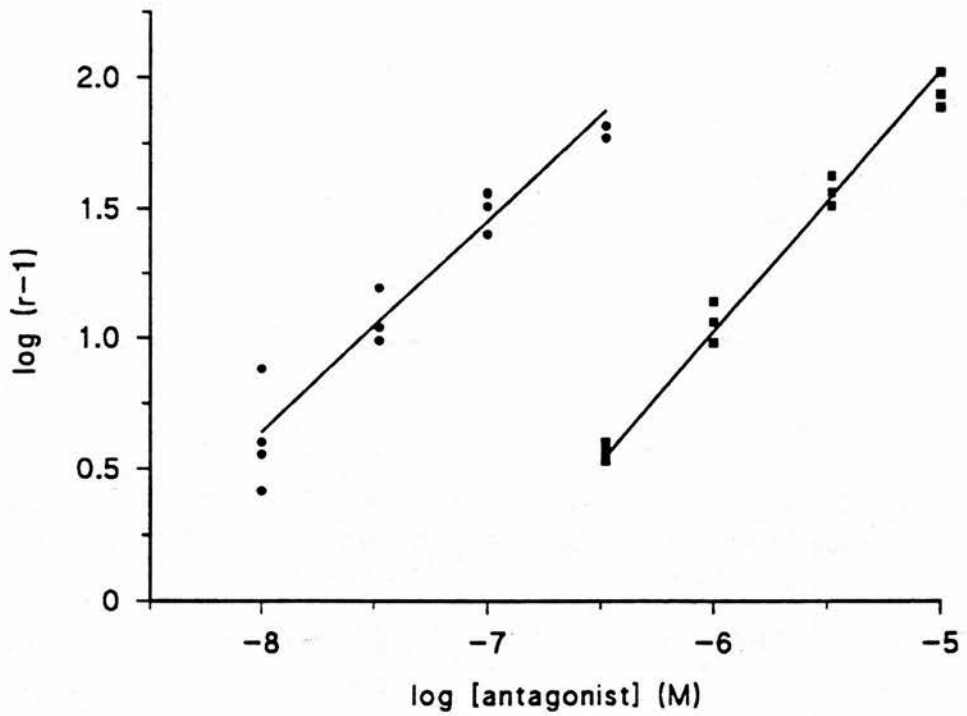

FIGURE 5.6 : Schild regression (least squares fit of $\log_{10}$ (r-1) *vs.* $\log_{10}$ [antagonist], where r = dose ratio) of the antagonism of AII-stimulated cortisol secretion by (●) saralasin and (■) losartan. Combined data from eight separate cell preparations.

TABLE 5.1

Antagonist	*pA2 (95% CL)*	*slope (95% CL)*	*Published pA2*
saralasin	8.79 (8.41-9.17)	0.81 (0.66-0.96)†	9.01∞
losartan	7.02 (6.89-7.15)	0.93 (0.85-1.02)	8.48∞*

†Slope significantly different from unity, **p<0.05**

Data from : ∞Liu *et al* (1992), *Chiu *et al* (1990)

TABLE 5.1 : Comparison of experimental pA_2 values for saralasin and losartan in zfr cells with previously published values.

FIGURE 5.7

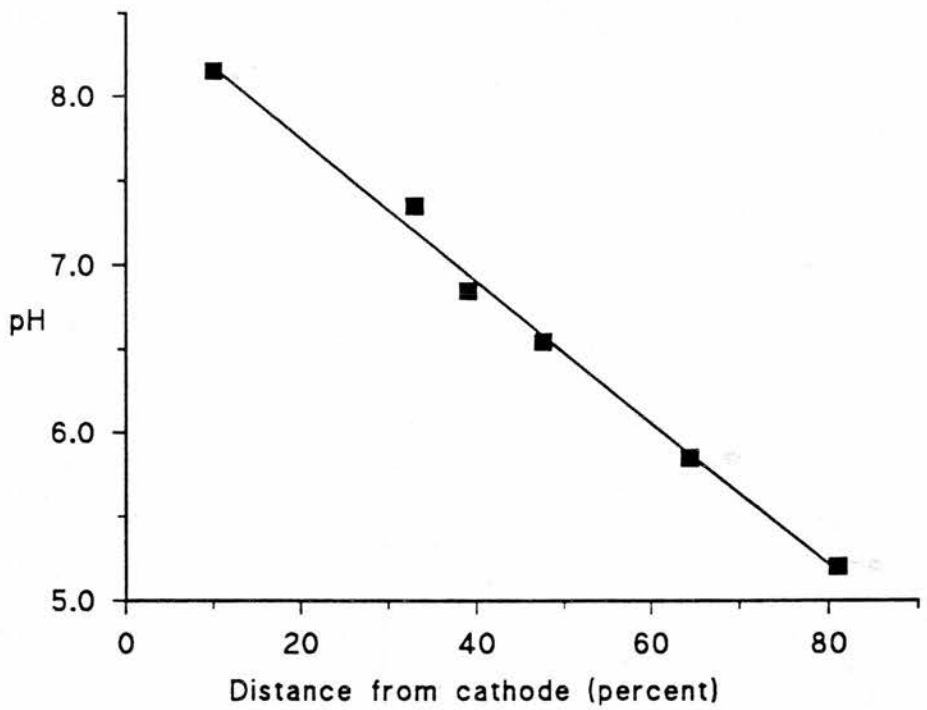

FIGURE 5.7 : Calibration of pH gradient across isoelectric focusing gels. Gels were loaded with protein markers of known isoelectric point and electrofocusing performed as described in Materials and Methods. The distance of each protein from the cathode is expressed as a percentage of the inter-electrode distance. Protein markers were (in order of decreasing Pi): lentil lectin - acidic, myoglobin - basic band, myoglobin - acidic band, carbonic anhydrase B (human), carbonic anhydrase B (bovine), β-lactoglobulin A.

FIGURE 5.8

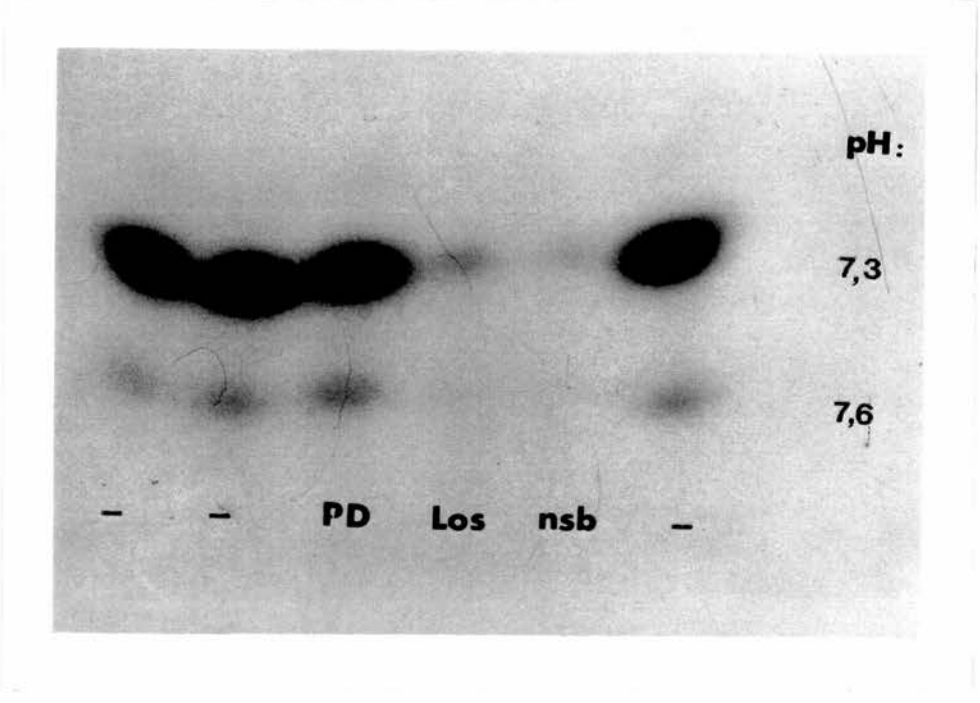

FIGURE 5.8 : Autoradiography of solubilised ^{125}I-AII binding proteins fractionated by isoelectric focusing. Crude membrane fractions from cultured bovine zfr cells were labelled with ^{125}I-AII in the presence or absence (-) of the following displacing agents: (los), losartan 10 μM; (PD), PD12317, 10 μM; (nsb), unlabelled AII, (10μM). The pH gradient is indicated on the right.

5.3 DISCUSSION

Bovine adrenocortical AII receptors were originally characterised according to the ability of peptide analogues of AII to inhibit the binding of ^{125}I-AII to adrenocortical microsomal membrane fractions (Glossman *et al,* 1974a,b; Catt & Aguilera, 1980). Although preliminary evidence for AII receptor heterogeneity was obtained (see section 1.6.1), definitive evidence for the existence of at least two subtypes of AII receptor awaited the development of the non-peptide antagonists losartan and PD123177, which bind selectively to AT_1 and AT_2 sites repectively. While both AT_1 and AT_2 sites are clearly present in rat, rabbit and human adrenal cortex (Chiu *et al,* 1989b; Whitbread *et al,* 1989; Chang & Lotti, 1991), bovine adrenocortical cells (unpurified) were reported to contain only the AT_1 site (Balla *et al,* 1991). By contrast, Ouali *et al* (1992) found both AT_1 and AT_2 binding sites in purified bovine zf cells. In the present chapter, the receptor subtype mediating AII-induced cortisol secretion from bovine zfr cells has been characterised using antagonist displacement, Schild and isoelectric focusing analyses.

As shown in Figures 5.1 - 5.3, AII-stimulated cortisol secretion and [^{3}H]phosphoinositol production were dose-dependently and completely inhibited both by saralasin and losartan, indicating the involvement of the AT_1 receptor in AII-stimulated steroidogenesis. Consistent with this hypothesis, the AT_2-selective antagonist PD123177 was without effect on either cortisol secretion or second messenger formation stimulated by AII. These findings are in agreement with those of Ouali *et al* (1992), who demonstrated AT_1 receptor-mediated cortisol secretion and PI turnover in bovine zf cells. This receptor subtype is also known to mediate AII-stimulated aldosterone secretion from cultured bovine zg cells (Balla *et al,* 1991) and from freshly isolated rat adrenal capsular cells (Chang & Lotti, 1989).

Closer examination of the inhibition curves shown in Figures 5.1 and 5.2 reveal two additional points requiring consideration. Firstly, while the curves for the antagonism of AII-stimulated cortisol secretion and [^{3}H]phosphoinositol production by saralasin were of the classical steep sigmoid shape, those for losartan were flatter, covering a larger antagonist concentration range (3-4 log units), possibly indicating complex antagonistic effects of losartan. Secondly, the potency of losartan in inhibiting the steroidogenic and second messenger responses to AII was lower than expected: transformation of the ID_{50} values for losartan according to the Cheng-Prusoff relationship (Cheng & Prusoff, 1973) would produce K_i values in the region of 10^{-7}M, while previous binding studies in other tissues reported K_i values for losartan of 10^{-9} - 10^{-8}M (see Timmermans *et al,* 1993). For

these reasons, the antagonism of AII-stimulated cortisol secretion by saralasin and losartan was further characterised by Schild analysis.

Saralasin produced parallel shifts of the AII dose-response curve without depressing the maximum response attainable by AII, consistent with competitive antagonism. The extrapolated pA_2 for saralasin (8.79) agreed well with previous reports in other systems (Table 5.1). Peptide analogues of AII have previously been reported to exhibit partial agonist properties, and saralasin has also been described as a non-competitive antagonist of AII-stimulated responses both *in vitro* (Wong *et al*, 1990; Freer *et al*, 1980) and *in vivo* (Chiu *et al*, 1988), as indicated by its non-parallel shifts of the dose response curve and depression of the upper asymptote. No such activity was observed for the antagonism of AII-stimulated cortisol secretion from bovine zfr cells. It is possible that the doses of saralasin employed (10 nM - 0.33 μM) were insufficient for partial agonism to be detected. However, depression of the maximum response to AII-induced contraction of rabbit aorta by saralasin is apparent at 3 nM saralasin (Wong *et al*, 1990). As reported by others (Liu *et al*, 1992), the slope of the Schild regression for saralasin was significantly lower than unity. This may be indicative of a saturable agonist uptake process which would result in underestimation of the dose ratios and produce a Schild regression of slope less than unity (Kenakin, 1985). Indeed, uptake of AII by cultured bovine adrenocortical cells has been reported, with AII internalisation occurring with a half-time of 10 min (Crozat *et al*, 1986). A further possibility is that saralasin acts in a non-competitive manner as reported by Wong *et al* (1990). In tissues with a large receptor reserve this would result in parallel shifts of the dose response curve without depression of the maximal response at low antagonist concentrations.

Consistent with previous reports (Liu *et al*, 1992; Chiu *et al*, 1990), losartan appeared to act as a classical competitive antagonist, producing parallel shifts of the dose response curve to AII without depressing the maximum response. Moreover, the resultant Schild regression was linear with slope not significantly different from unity. However, the extrapolated pA_2 value for losartan (7.02) corresponds to an affinity some 30 times lower than that previously reported for the antagonism of AII-mediated contraction of rabbit aorta (pA_2 = 8.48, Table 5.1). This observation is in agreement with the low IC_{50} values obtained for losartan (FIgures 5.1 and 5.2). Although differences in assay conditions might be expected to lead to a small difference in pA_2 values for the same receptor, pA_2 measurements are generally in close agreement between laboratories (Schild, 1949).

It seems likely that this discrepancy between the pA_2 values for losartan in bovine zfr cells compared with rabbit smooth muscle represents a true difference since a reduced

potency of losartan in bovine adrenal cortex has been reported in previous binding studies: losartan was around ten-fold less potent in inhibiting ^{125}I-AII binding to bovine, than to rat, adrenocortical AT_1 receptors (Balla *et al*, 1991). A similarly low K_i for losartan was reported in cultured bovine zf cells (Ouali *et al*, 1992). It is unlikely that this difference can be accounted for by differences in the AT_1 receptor microenvironment between species: comparison of the ^{125}I-AII displacement curves in membrane fractions of COS-7 cells transfected with AT_1 receptor cDNA from bovine adrenal cortex (Sasaki *et al*, 1991) or rat vascular smooth muscle (Murphy *et al*, 1991) reveal a reduced potency of losartan at the adrenal ($IC_{50} \cong 3x10^{-7}M$) compared to the vascular ($IC_{50} \cong 5x10^{-9}M$) receptor. Although this difference has been attributed to differences in the assay conditions between laboratories (Catt & Abbot, 1991), the pA_2 values obtained in the present study, together with the binding data discussed above, raise the possibility that losartan may recognise a distinct subtype of AT_1 receptor in bovine adrenal cortex with different pharmacological characteristics to those AT_1 receptors previously described. This hypothesis would accord with previous functional studies in adrenal and vascular tissues: although AII acts via the AT_1 receptor in both bovine adrenal cortex and rat vascular smooth muscle (Balla *et al*, 1991; Ouali *et al*, 1992; Chiu *et al*, 1991), the AII peptide analogues $(sar)_5$-AII and $(pro)_3$-AII were weak agonists/partial antagonists of AII-mediated responses in bovine adrenocortical cells, but acted as full antagonists in rat aortic smooth muscle cells (Capponi *et al*, 1991).

Although several subtypes of losartan-sensitive AII receptors have been cloned (section 1.6.5), the receptor mediating AII-induced cortisol secretion from bovine zfr cells does not appear to resemble any previously characterised receptor. The cloned AT_{1A} and AT_{1B} receptor subtypes isolated from rat (Kakar *et al*, 1992) and murine (Sasamura *et al*, 1992) tissues bound losartan with equal and high (low nanomolar) affinity. Moreover, the novel AII receptor recently isolated from from the rat adrenal glomerulosa does not resemble the bovine zfr recepor since this subtype (termed AT_3) exhibited a higher affinity for losartan than the previously characterised AT_1 receptors (Sandberg *et al*, 1992).

In an attempt to further characterise the bovine zfr AII receptor, membrane fractions from zfr cells were labelled with ^{125}I-AII, fractionated by isoelectric focusing, and the bound label visualised by autoradiography. This technique, which separates proteins according to differences in charge rather than molecular wight, has previously been shown to separate multiple isoforms of oestrogen receptor (Baker *et al*, 1992), epidermal growth factor receptor (Wenisch *et al*, 1992), as well as DTT-sensitive and -insensitive (ie. AT_1 and AT_2) binding proteins from rat tissues (Jimenez *et al*, 1991). Following isoelectric focusing, two distinct bands were visible, migrating to isoelectric points (p*I*) of 7.3 and

7.6. Jimenez *et al* (1991) resolved up to four separate AII binding proteins in rat tissues by isoelectric focusing, of p*I* between 6.3 and 6.8. Thus the receptor isoforms identified in bovine zfr cells do not appear to correlate with those present in the rat. Although the focusing conditions used in the present study were similar to those of Jimenez *et al* with respect to time, voltage and current, these authors did not state whether such conditions were sufficient to allow ensure migration to the *true* p*I*, or whether they simply allowed separation of the different receptor isoforms. Furthermore, a number of differences exist between the two methodologies with respect to the carrier ampholyte used (pharmalyte *vs* ampholine), method of gel polymerisation (ammonium persulphate *vs* riboflavin/UV light), electrode solutions and focusing temperature. Since all of these conditions are known to affect the apparent equilibrium p*I* (Crambach & Baumann, 1976), comparison of the p*I* values obtained in this study with those of Jimenez *et al* may not be valid. It should also be noted that while the present study utilised membrane fractions prepared from a purified zfr cell preparation, the study of Jimenez *et al* identified AII binding proteins using membranes prepared from a whole gland homogenate. It is likely that this preparation contained membrane particles from a variety of cell types such as vascular tissue, red blood cells and mast cells, all of which may contain AII receptors.

In an attempt to identify the two ^{125}I-AII binding proteins present in bovine zfr cells, incubations were performed in the presence of selective displacing agents. Binding of ^{125}I-AII to both proteins was inhibited in the presence of losartan, but was not affected by PD123177. The absence of PD123177-displaceable binding is in agreement with previous binding studies which either failed to detect AT_2 binding sites (Balla *et al*, 1991), or found a very low density of AT_2 sites (Ouali *et al*, 1992), in bovine adrenocortical cells. While it is tempting to speculate that the two losartan-sensitive bands represent distinct subtypes of AT_1 receptor, a number of possibilities could account for their presence. It is possible that one of the bands represents a proteolytic breakdown product of the AT_1 receptor, although whether such a molecule would retain its ^{125}I-AII binding properties is doubtful. Alternatively, the two bands may represent glycosylated and de-glycosylated forms of the same receptor: sequence analysis of the cloned AT_1 receptor from bovine adrenal cortex showed three potential N-gycosylation sites (Sasaki *et al*, 1991). Furthermore, the purified rat liver AT_1 receptor was shown to be heavily glycosylated (Desarnaud *et al*, 1993). This possibility seems unlikely however, since the purified AII receptor from various tissues has a molecular weight corresponding to the glycosylated form of the receptor (ca. 65 kD) (Guillemette *et al*, 1987; Guillemette & Escher, 1983; Desarnaud *et al*, 1993), and deglycosylation of purified AT_1 receptors required quite harsh chemical or enzymatic procedures (Desarnaud *et al*, 1993). Thirdly,

the bands may represent phosphorylated and de-phosphorylated forms of the receptor, or sulphydryl-oxidised/reduced forms.

The possibility that bovine zfr cells contain two distinct subtypes of AT_1 receptor, analogous to other AII target tissues (Kakar *et al,* 1992; Iwai & Inagami, 1992; Sandberg *et al,* 1992), would accord with the reported differential potency of losartan in inhibiting AII-stimulated responses in these cells. Losartan was around 60-fold more potent in inhibiting AII-stimulated [^{3}H]thymidine incorporation into bovine zfr cells than in inhibiting the steroidogenic response to AII (Clyne *et al,* 1993).

Although the present results are clearly insufficient to confirm or exclude this possibility, such a hypothesis would be in agreement with the available data. The presence of two distinct AT_1 receptors, each subserving the same response (ie. linked to PLC) but with different affinities for losartan, would be expected to result in a biphasic antagonist inhibition curve. However, if the affinities of the two receptors for losartan were not greatly different (ca. 1 log unit difference), then the two phases of the inhibition curve would appear to merge, forming a more shallow monophasic curve such as those presented in Figures 5.1 and 5.2. By contrast, saralasin, which exhibits equal affinity for all known AII receptors (Timmermans *et al,* 1991), would produce a steep classical monophasic inhibition curve. Moreover, such a situation would not necessarily be evident from Schild analysis, which could produce a linear Schild regression of unit slope for losartan (Kenakin, 1985). This hypothesis should be open to experimental testing, since in the situation described above, the antagonism of a heterogenous receptor population by losartan would be expected to result in agonist-dependent Schild regressions (Kenakin, 1985). Furthermore, the densitometric analysis of the effect of increasing concentrations of losartan on the AII binding proteins separated by isoelectric focusing would provide estimates of the affinity of losartan for each binding site.

In conclusion, the results presented in this chapter establish that AII-stimulated cortisol secretion from bovine zfr cells occurs through activation of the AT_1 receptor. However, this receptor appears to exhibit different characteristics from the previously characterised AT_1 receptor in other tissues, based on the low potency of losartan in zfr cells. In addition, isoelectric focusing experiments have raised the possibility that bovine zfr cells contain two distinct AT_1 receptors, and the address of this question should prove a fruitful area of future research.

CHAPTER 6

HORMONE-SENSITIVE AND -INSENSITIVE POOLS OF PHOSPHOINOSITIDES IN BOVINE ZFR CELLS STIMULATED WITH ACETYLCHOLINE AND ANGIOTENSIN II

6.1 INTRODUCTION

Over the past thirty years considerable advances have been made in the understanding of receptor-mediated PtdIns metabolism. One area which still remains controversial, however, is the concept that discrete pools of inositol phospholipid may be involved in signal transduction, ie. that cells may contain hormone-sensitive and -insensitive pools of phosphoinositides. That only a small proportion of total cellular lipid is accessible to hormone-stimulated catabolism was first suggested by Hokin and Hokin (1964), based on their studies of ^{32}P metabolism in the avian salt gland. Subsequent studies have largely been conducted using the rat mammary tumour (WRK-1) cell line. Thus Monaco (1982) found that vasopressin could stimulate loss of ^{32}P from PtdIns fractions of ^{32}P labelled WRK-1 cells if, and only if, vasopressin was itself included during the ^{32}P labelling period. This vasopressin-sensitive pool of phosphoinositides was shown to account for around 17% of total cellular PtdIns (Monaco, 1982). Subsequent studies designed to characterise the hormone-sensitive pool of phosphoinositides in WRK-1 cells found that it was discrete, in that no transfer of PtdIns between hormone sensitive and -insensitive pools occurred (Monaco & Woods, 1983). Furthermore, PtdIns(4,5)P_2 derived from hormone-sensitive PtdIns was itself accessible for signal transduction, whereas that derived from hormone-insensitive PtdIns was not (Koreh & Monaco, 1986).

A variety of cells from different tissues/species have now been proposed to contain multiple pools of phosphoinositides, including rabbit and horse platelets (Vickers & Mustard, 1986; Billah & Lapetina, 1982), rat liver, pancreas and brain (Putumraj & Slaby, 1982; Rana *et al*, 1986; LaBarca *et al*, 1985), rabbit kidney (Schwartzman *et al*, 1981) and human neutrophils and erythrocytes (Muller *et al*, 1986; Cockcroft & Allan, 1984; King *et al*, 1987).

The possible occurance of distinct subpools of phosphoinositides in adrenocortical cells has been little studied. However, Bird *et al* (1992c) obtained evidence for the presence of two metabolically distinct hormone-sensitive phosphoinositide pools in [^{3}H]inositol labelled bovine zfr cells. When stimulated with AII, [^{3}H]inositol phosphate production occurred through turnover of a small (30%) pool of [^{3}H]phosphoinositides maintained through utilisation of cytosolic [^{3}H]inositol for up to 30 min. After this time, when cytosolic [^{3}H]inositol became depleted, a second (45%) pool of phosphoinositides was metabolised until it too became depleted after a further 60 min (Bird *et al,* 1992c).

Given this evidence for multiple phosphoinositide pools in bovine zfr cells, an important question is whether a common pool is broken down in response to different agonists, or whether different agonists stimulate the catabolism of discrete subpools of phosphoinositides. In this chapter, evidence is presented to suggest that in bovine zfr cells, ACh and AII stimulate the breakdown of a common phosphoinositide pool.

6.2 RESULTS

6.2.1 Correlation between acetylcholine- and angiotensin II-stimulated cortisol secretion and PLC activity

Figures 6.1 and 6.2 show dose response curves for the secretion of cortisol and production of [^{3}H]phosphoinositols (in cells labelled to steady state with [^{3}H]inositol) in response to ACh and AII respectively. For both ACh and AII, the dose-response curves for cortisol secretion and phosphoinositol production fell in a similar dose range (10^{-7} - 10^{-4}M for ACh (Figure 6.1), 3 x 10^{-11} - 3 x10^{-9}M for AII, Figure 6.2). In addition, the EC_{50} values for each response were similar: ACh stimulated cortisol secretion and phosphoinositol production with EC_{50} values of 1.81 x 10^{-6}M and 1.06 x 10^{-6}M respectively, while the EC_{50} values for AII-stimulated steroidogenesis and second messenger formation were 1.3 x 10^{-10}M and 2.45 x 10^{-10}M. Plotting the magnitudes of the cortisol and phosphoinositol responses as a function of each other revealed a highly significant correlation between PLC activation and cortisol secretion for each agonist (Figure 6.3): the correlation coefficients (r) for ACh and AII were 0.972 and 0.964 respectively (both $p<0.01$). Moreover, the gradients of the two regression lines were not significantly different (8.32 $\pm$ 1.21 for ACh and 8.34 $\pm$ 1.07 for AII)

6.2.2 Partial additivity of acetylcholine- and angiotensin II-stimulated cortisol secretion and phosphoinositol production

In order to determine if any additivity of effect occurred on co-addition of ACh and AII, the effect of submaximal (10^{-9}M) and maximal (10^{-7}M) doses of AII on ACh-stimulated phosphoinositol production and cortisol secretion was investigated. Examples of such experiments are shown in Figures 6.4 and 6.5. Responses were termed "additive" if the incremental response to ACh was the same magnitude in the presence and absence of AII. In five out of eight experiments, ACh (10^{-6}M) stimulated a significant ($P<0.05$) increase in phosphoinositol production over basal levels in cells pre-labelled to steady state with [^{3}H]inositol, while phosphoinositol production in the presence of ACh (10^{-4}M) was significantly increased ($p<0.01$) in all experiments (n=8) (Figure 6.4, upper panel). When repeated in the presence of AII (10^{-9} M) the effects of ACh (10^{-4}M) on phosphoinositol production were partially additive. The extent of additivity ranged from 0% to 100% between experiments (0% additivity representing no effect of ACh over the AII response, 100% additivity representing an equal incremental increase in the phosphoinositol response to ACh in the presence and absence of AII). In two out of eight experiments the effects of ACh and AII on phospholipase C activity were fully additive;

FIGURE 6.1

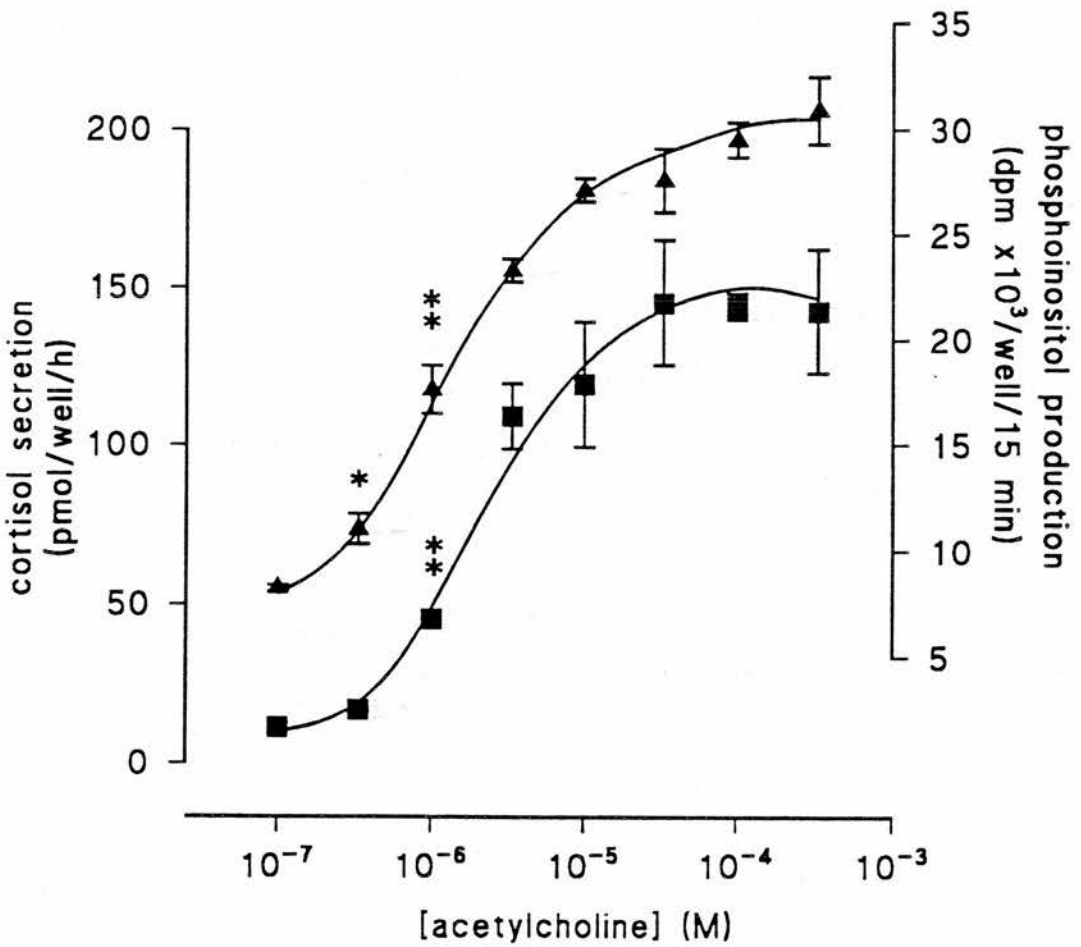

FIGURE 6.1 : Dose response curves for acetylcholine-stimulated cortisol secretion (■) and phosphoinositol production (▲) from bovine zfr cells. Data points are the mean ± SD of triplicate measurements from one experiment. Significant stimulation relative to control values is indicated at *$p<0.05$, **$p<0.01$.

FIGURE 6.2

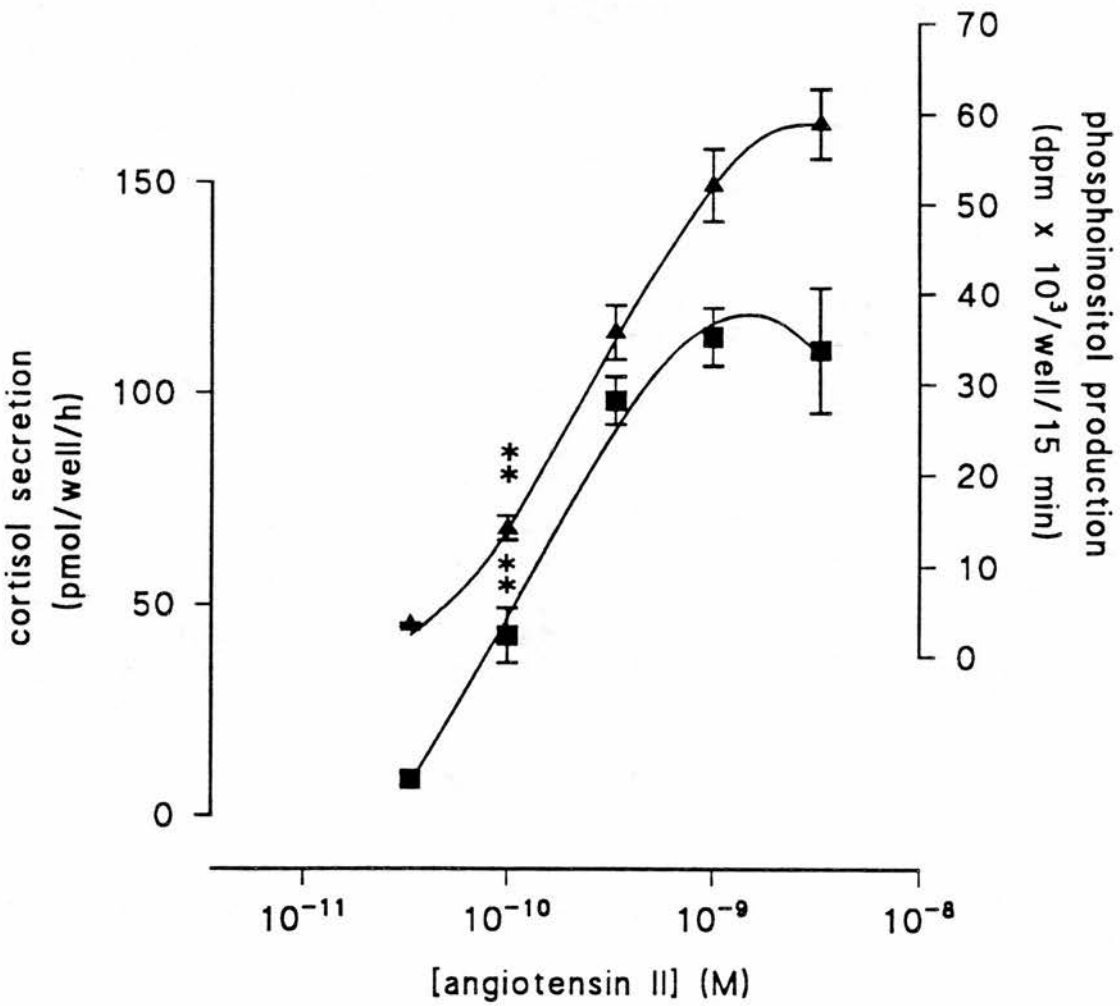

FIGURE 6.2 : Dose response curves for angiotensin II-stimulated cortisol secretion (■) and phosphoinositol production (▲) from bovine zfr cells. Data points are the mean ± SD of triplicate measurements from one experiment. Significant stimulation relative to control values is indicated at *p<0.05, **p<0.01.

FIGURE 6.3

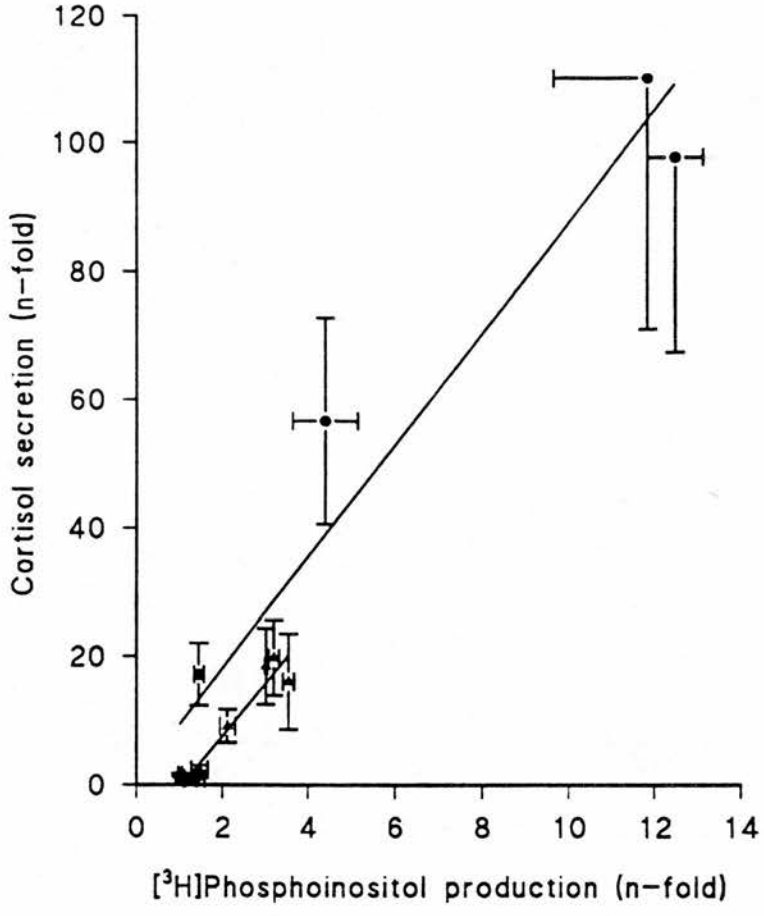

FIGURE 6.3 : Correlation between acetylcholine- and angiotensin II-stimulated cortisol secretion and phosphoinositol production in bovine zfr cells. Cortisol secretion and phosphoinositol production were measured in response to acetylcholine (10^{-9}-10^{-3}M) (▲) and angiotensin II (10^{-11}-10^{-7}M) (●). Results are expressed as n-fold stimulation ratios and show the linear relationship between cortisol secretion and phospholipase C activity (as measured by the accumulation of total labelled phosphoinositol headgroups) for both agonists. Each data point represents the mean ± s.e.m. of data from 3-6 experiments, each performed in triplicate.

in five out of eight experiments the effect of both agonists on phospholipase C activity was greater than the effects of either agonist alone ($p<0.05$) (ie. partially additive), while in one experiment, no further increase in phosphoinositol production over the AII response was observed in response to ACh.

The effects of ACh and AII on cortisol secretion were also assessed (Figure 6.4, lower panel). As before, variable results were obtained, with additivity ranging from 0%-100%. Full additivity in the responses to ACh (10^{-4}M) and AII (10^{-9}M) occurred in two out of eight experiments, partial additivity in three out of eight experiments, and in the remaining three experiments, no further increase in cortisol secretion over the response to AII occurred in response to ACh.

The effects of ACh on phospholipase C activity and cortisol secretion stimulated by a maximal dose of AII (10^{-7}M) were also studied (Figure 6.5). In eight out of eight experiments, no significant increase in PLC activity stimulated by AII (10^{-7}M) occurred on co-administration of ACh (10^{-6}M, 10^{-4}M). Similarly, in seven out of eight experiments in which cortisol secretion was measured as the end response, no effect of ACh on AII (10^{-7}M)-stimulated cortisol secretion was observed. In one experiment, the effects of ACh (10^{-4}M) and AII (10^{-7}M) were partially additive ($p<0.05$).

6.2.3 Effects of acetylcholine and Mn^{2+} pre-incubation on AII-stimulated redistribution of radioactivity between phosphoinositides, phosphoinositols and free inositol

Cells were labelled for 6 h with [^{3}H]inositol in the presence or absence of ACh (10^{-4}M) or $MnCl_2$ (10^{-3}M) (see section 2.2.5ii), and subsequently challenged with AII (10^{-7}M) in the presence of lithium for 15 min or 2h. The resultant distribution of radioactivity between phosphoinositide, free inositol and phosphoinositol fractions in such cells is shown in Figures 6.6 and 6.7 respectively. Previous studies have shown that under these labelling conditions, the majority of the radiolabel is found in the free inositol fraction (Bird *et al,* 1992c).

In control cells (no prestimulation during the labelling period), subsequent acute (15 min) stimulation with AII resulted in increased turnover of the phosphoinositides. This was apparent from the increased accumulation of label in both the [^{3}H]phosphoinositide and [^{3}H]phosphoinositol fractions, and corresponding decrease in free [^{3}H]inositol (all $p<0.01$) (Figure 6.6). Labelling in the presence of ACh (10^{-4}M) for 1 h increased the incorporation of label into the [^{3}H]phosphoinositide fraction ($p<0.05$). However, when

FIGURE 6.4

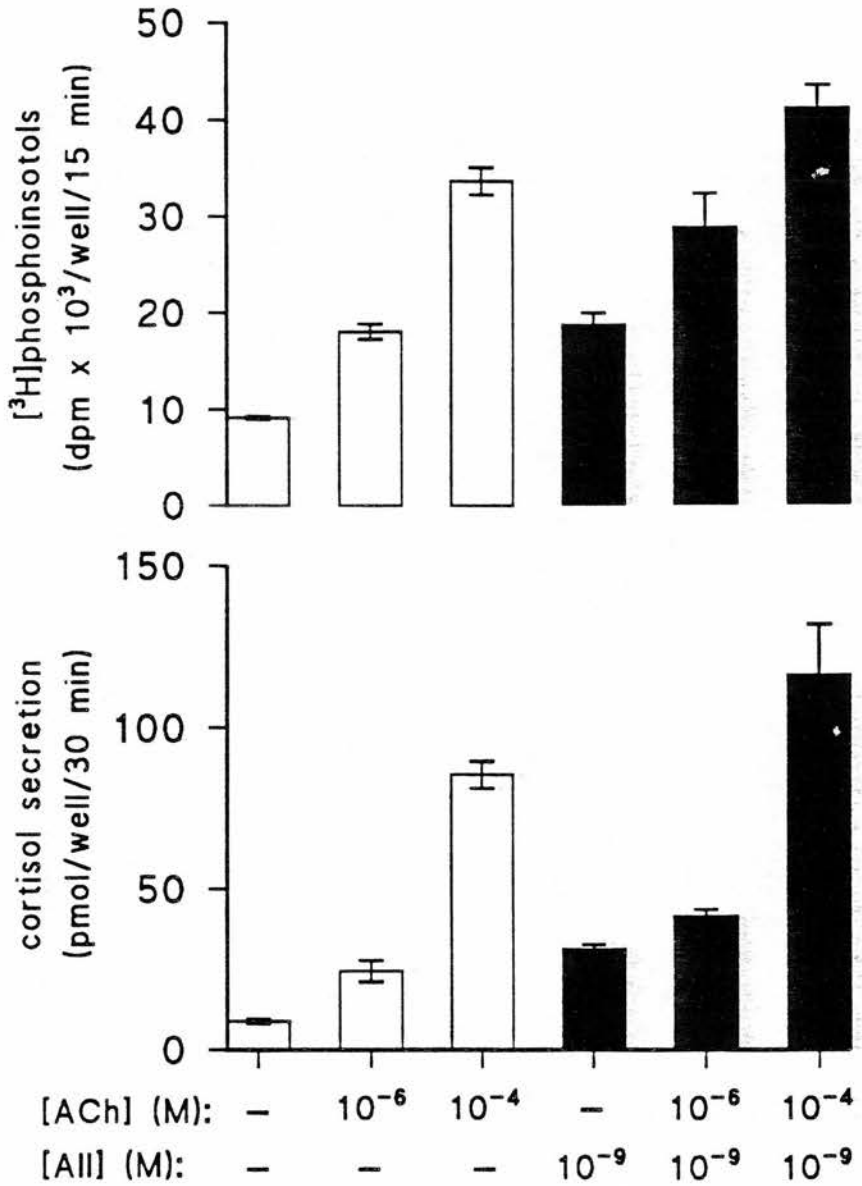

FIGURE 6.4 : Effect of acetylcholine on phosphoinositol production (upper panel) and cortisol secretion (lower panel) from bovine zfr cells either alone (open bars) or in combination with a sub-maximal (10^{-9}M) dose of angiotensin II (solid bars). Data are the mean $\pm$ SD of triplicate determinations from a single experiment which was repeated on eight occasions with varying results (see text for details).

FIGURE 6.5

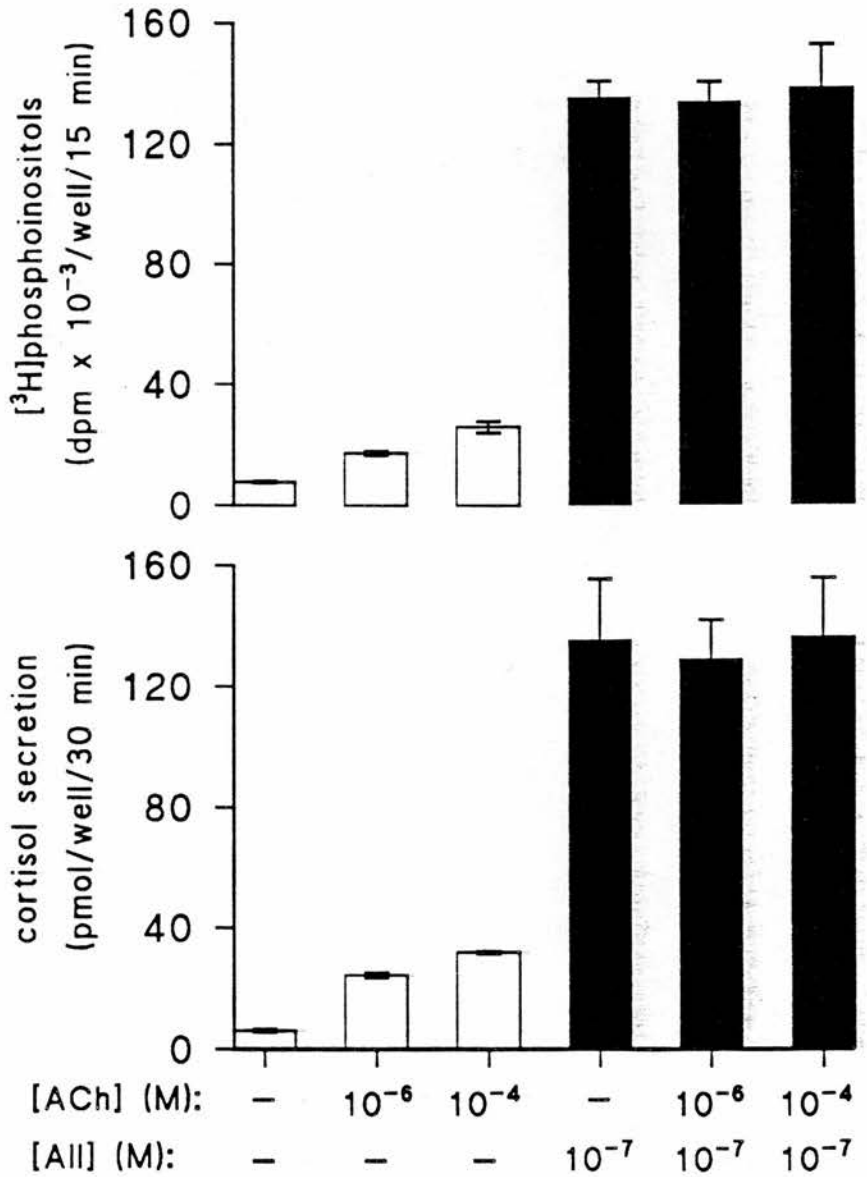

FIGURE 6.5 : Effect of acetylcholine on phosphoinositol production (upper panel) and cortisol secretion (lower panel) from bovine zfr cells either alone (open bars) or in combination with a maximal (10^{-7}M) dose of angiotensin II (solid bars). Data are the mean ± SD of triplicate determinations from a single experiment which was repeated on eight occasions with similar results.

such ACh-pretreated cells were subsequently stimulated with AII (10^{-7}M), the maximal incorporation of label into [^{3}H]phosphoinositides was not significantly different from that observed in control cells, indicating that the same steady state was achieved on acute (15 min) stimulation with AII whether cells had previously been stimulated with ACh or not. Furthermore, the magnitude of the [^{3}H]phosphoinositol response to AII was not significantly different in control and ACh-pretreated cells (Figure 6.6, lower panel)

Cells which had been pre-labelled with [^{3}H]inositol in the presence of $MnCl_2$ exhibited an increased incorporation of label into the [^{3}H]phosphoinositide fraction and a correspondingly reduced labelling of the free [^{3}H]inositol fraction compared with control cells (both $p<0.05$). However, on subsequent acute stimulation with AII, the incremental increases in the labelling of the [^{3}H]phosphinositides and [^{3}H]phosphoinositols, and the incremental loss of [^{3}H]inositol, were not significantly different from those measured in control cells.

The effects of ACh and $MnCl_2$ pre-treatment were also investigated in cells subsequently stimulated chronically with AII (10^{-7}M, 2 h) (Figure 6.7). In control cells (no pre-treatment during labelling), chronic (2 h) stimulation with AII (10^{-7}M) resulted in a loss of radioactivity from the [^{3}H]phosphoinositide and free [^{3}H]inositol fractions (both $p<0.05$). As would be expected on prolonged stimulation in the presence of LiCl, a correspondingly large increase in the level of [^{3}H]phosphoinositols occurred on chronic AII stimulation ($P<0.01$). Labelling in the presence of ACh increased the incorporation of label into the [^{3}H]phosphoinositide fraction ($p<0.01$). However, the levels to which [^{3}H]phosphoinositide labelling and free [^{3}H]inositol levels dropped on subsequent chronic stimulation with AII were unaffected by ACh pre-treatment. In addition, the magnitide of the [^{3}H]phosphoinositol response to AII was not significantly different in control and ACh-pre-treated cells (Figure 6.7, lower panel). In contrast, when $MnCl_2$ pre-treated cells were subsequently subjected to chronic (2 h) stimulation with AII, the elevated level of [^{3}H]phosphoinositide labelling was decreased by the same increment, but not to the same level, as that observed in control cells. In addition, the magnitude of the [^{3}H]phosphoinositol response to AII was reduced in $MnCl_2$ pre-treated cells compared to that observed in both control and ACh pre-treated cells (both $p<0.01$).

FIGURE 6.6

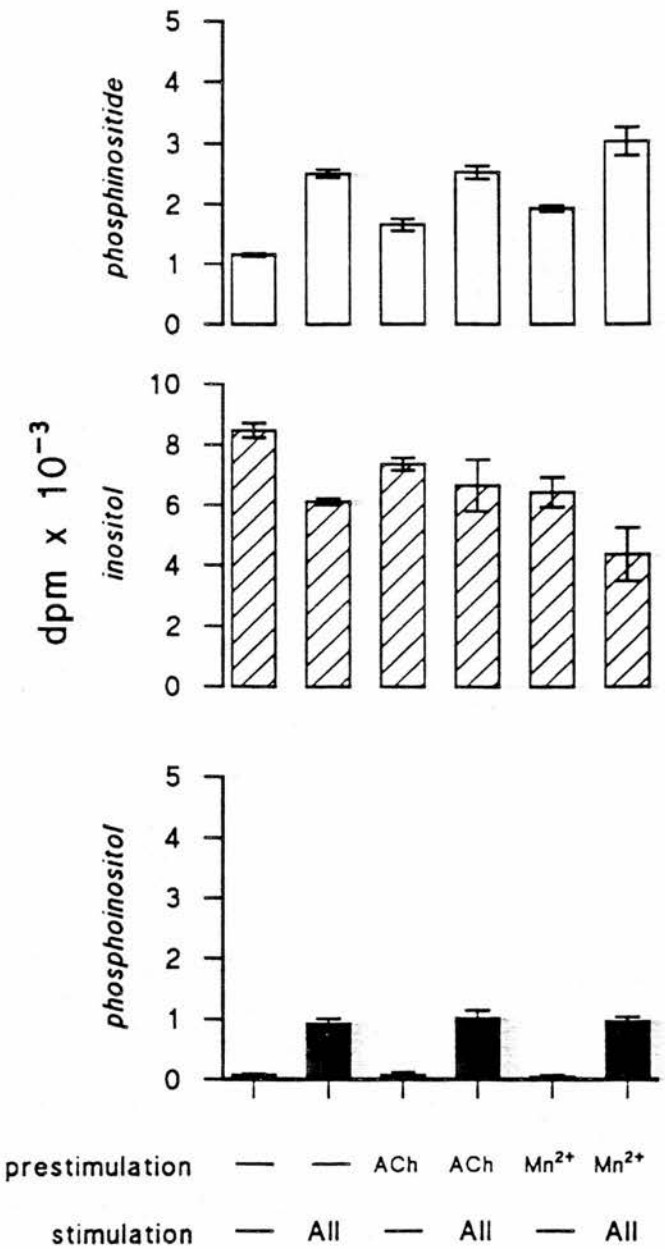

FIGURE 6.6 : Effect of acetylcholine and manganese pre-stimulation on subsequent acute angiotensin II-stimulated redistribution of radioactivity between [^{3}H]phosphoinositide, [^{3}H]inositol and [^{3}H]phosphoinositol fractions of pre-labelled bovine zfr cells. Cells were labelled with [^{3}H]inositol for 6 h in the presence of $MnCl_2$ (10^{-3}M) or acetylcholine (10^{-4}M) as described in section 2.5.1, and subsequently incubated in the presence or absence of angiotensin II (10^{-7}M) for 15 min (in medium containing LiCl, 10mM). The distribution of radioactivity between the three [^{3}H]inositol-labelled fractions is shown. Values are the mean $\pm$ SD of triplicate determinations from one representative experiment of three similar experiments.

FIGURE 6.7

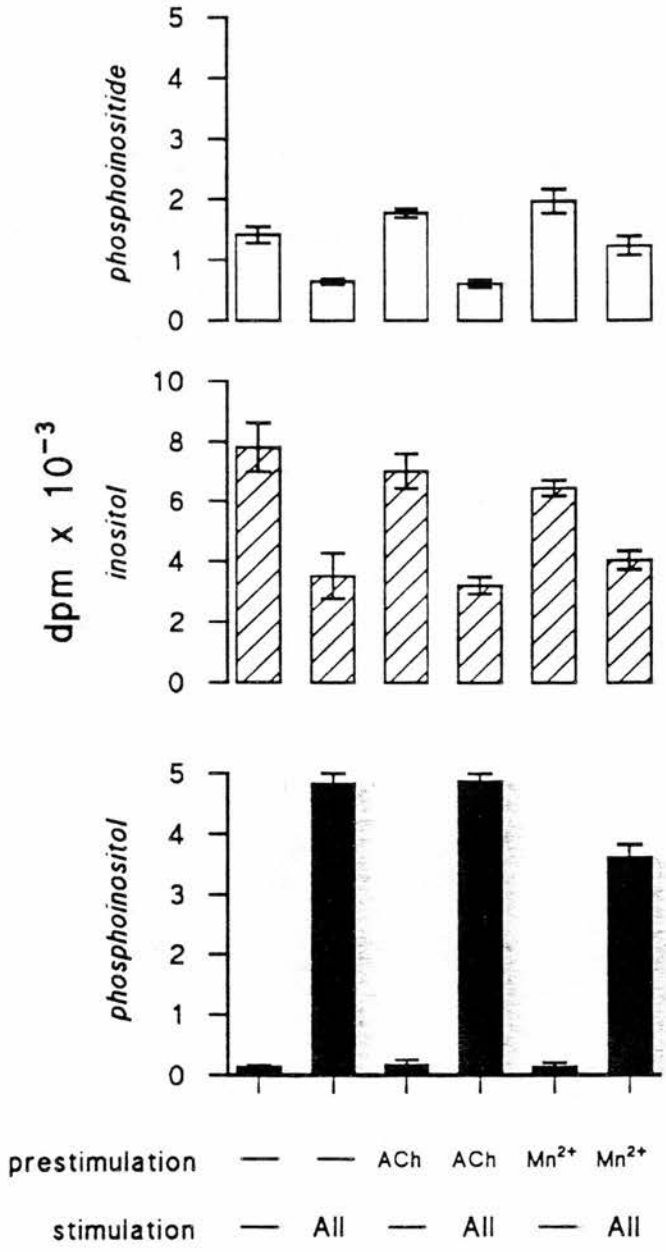

FIGURE 6.7 : Effect of acetylcholine and manganese pre-stimulation on subsequent chronic angiotensin II-stimulated redistribution of radioactivity between [^{3}H]phosphoinositide, [^{3}H]inositol and [^{3}H]phosphoinositol fractions of pre-labelled bovine zfr cells. Cells were labelled with [^{3}H]inositol for 6 h in the presence of $MnCl_2$ (10^{-3}M) or acetylcholine (10^{-4}M) as described in section 2.5.1, and subsequently incubated in the presence or absence of angiotensin II (10^{-7}M) for 2 h (in medium containing LiCl, 10mM). The distribution of radioactivity between the three [^{3}H]inositol-labelled fractions is shown. Values are the mean ± SD of triplicate determinations from one representative experiment of three similar experiments.

6.3 DISCUSSION

Previous studies have demonstrated many similarities in the cellular effects and mechanisms of action of ACh and AII in bovine adrenocortical zfr cells. Neither agonist has any acute effect on cAMP levels, whereas both stimulate a hormone-sensitive phospholipase C (Walker *et al,* 1990; Bird *et al,* 1989). ACh and AII also stimulate dose-dependent increases in intracellular calcium concentration, and both agonists appear to mobilise a common intracellular calcium pool (Walker *et al,* 1991). These similarities would suggest that ACh and AII stimulate steroidogenesis through a common mechanism in bovine zfr cells. It is not clear, however, whether ACh and AII receptors are coupled to distinct subpools of PLC, which in turn may act on separate subpools of phosphoinositides, or whether a single pool of enzyme acts on a common pool of phosphoinositides. This is particularly relevant given the existence of at least two distinct hormone-sensitive pools of phosphoinositides in these cells (Bird *et al,* 1992c).

For measurement of PLC activity, cells were prelabelled with [^{3}H]inositol for 48 h. Previous studies have established that the phosphoinositides of bovine zfr cells label to steady-state by this time. Therefore, any increase in [^{3}H]phosphoinositol production in the presence of lithium is a reflection of PLC activity (Bird *et al,* 1989,1990b). Figures 6.1 and 6.2 illustrate the close correspondence between ACh- and AII-stimulated PLC activity and cortisol secretion. Both agonists stimulated [^{3}H]phosphoinositol production and cortisol secretion with similar dose dependencies. A strong correlation between these two responses was revealed (Figure 6.3). Moreover, the gradients of the regression lines for ACh and AII were near identical (8.32 ± 1.21 and 8.34 ± 1.07 respectively). Thus the relationship of PLC activation to cortisol secretion for each agonist appears to be identical, consistent with a common mechanism of action.

Bird *et al* (1990a) suggested that ACh and AII activate the same PLC in bovine zfr cells, based on the relationship between phosphoinositol labelling in response to ACh and AII in normal and low calcium media. In order to confirm this hypothesis, the possible additive effects of ACh and AII on PLC activity and cortisol secretion were examined. If both agonists share a common mechanism of action, but stimulate separate pools of PLC, then the effects of both sub-maximal and maximal doses of each agonist would be expected to be additive. If however a single pool of enzyme is stimulated by both agonists then there are two possible outcomes. In the event that AII receptors are available in excess and can fully stimulate the available PLC, then ACh would be expected to have no further effect on PLC activity or cortisol secretion stimulated by a maximally effective dose of AII. Alternatively, if AII receptors are limiting, then the

effects of ACh and AII may still be additive at maximal doses of AII. That AII-stimulated steroidgenesis is unimpaired by a substantial loss of AII receptors (Penhoat *et al,* 1988) suggests that AII receptors are present in excess over the available PLC. Therefore, if both agonists stimulate a common pool of enzyme, then their effects would be expected to be additive at sub-maximal, but not maximal, doses of AII.

The situation in bovine zfr cells appeared to be more complex than that described above, in that full additivity was rarely observed at submaximal doses of AII. In most experiments, for both PLC activity and cortisol secretion, the effects of ACh and AII were only partially additive. The interpretation of these results are probably complicated by the rapid desensitisation of the ACh response. Pre-incubation with ACh (10^{-4}M) for 30 min significantly reduced, and, in some experiments, completely abolished subsequent responses to ACh (Chapter 4). Although it is not known if the ACh and AII responses are subject to heterologous desensitisation, such a phenomenon has been reported to result in partial additivity of bradykinin and vasopressin responses in WRK-1 cells (Monaco *et al,* 1990). Thus, the possibility of heterologous desensitisation in zfr cells cannot be discounted.

The effects of ACh and AII at maximal doses were not additive with respect to PLC activity and cortisol secretion (Figure 6.5). However, two points require consideration. Firstly, if heterologous desensitisation does occur, as suggested above, then the desensitising effects would be expected to be greater at maximal agonist concentrations than at submaximal doses. This could result in a lack of additivity even if separate pools of PLC were stimulated by ACh and AII. Secondly, the standard deviations of the PLC and cortisol responses to AII (10^{-7}M, Figure 6.5) were of a similar magnitude to the incremental increases in response to ACh. Thus, the inherent variability of the cell preparation could mask any further effects of ACh at maximal concentrations of AII. Thus, although the additivity data is not inconsistent with the stimulation of a common pool of PLC by ACh and AII, firm conclusions cannot be drawn from the data set.

In order to investigate the phosphinositide pools labelled in response to ACh and AII, [^{3}H]phosphoinositide breakdown and resynthesis were examined in cells pre-labelled with [^{3}H]inositol for 6 h. Zfr cells labelled in this way contain large reserves of [^{3}H]inositol in the cytosol, but incorporate only small amounts of label into the phosphoinositides (Bird *et al,* 1992c). Since cells are not labelled to isotopic equilibrium, resynthesis of phosphoinositides (as a consequence of agonist stimulated phosphoinositide turnover) results in increased labelling of the phosphoinositide pool. In the presence of LiCl, which prevents phosphoinositol recycling into inositol, AII

stimulates incorporation to a new steady state within 15 min. After 30 min of stimulation in the presence of Li, depletion of cytosolic [^{3}H]inositol results in the breakdown of newly synthesised phosphinositides without resynthesis. Thus, at stimulation times longer than 30 min, breakdown of this newly synthesised lipid can be observed (Bird *et al*, 1992c).

The effect of ACh and $MnCl_2$ pre-stimulation on the redistribution of label in response to subsequent acute (15 min) AII stimulation (Figure 6.6) shows that although pre-stimulation with ACh increased [^{3}H]phosphoinositide labelling, the maximal incorporation of label into the phosphoinositide pool was unaffected by ACh pre-treatment. This is consistent with the stimulation by ACh of incorporation of [^{3}H]inositol into the same hormone-sensitive phosphoinositide pool as that stimulated by AII. If ACh had stimulated labelling of a different pool than that labelled in response to AII, then the label incorporation into phosphoinositides would be greater with ACh preincubation than without. Consistent with this hypothesis, the magnitude of the phosphinositol response to acute AII stimulation was unaffected by ACh pre-stimulation during labelling.

The effects of [^{3}H]inositol labelling were also studied in the presence of $MnCl_2$. Mn^{2+} treatment has been shown in other cells to stimulate labelling by both PtdIns synthase activity and by inositol headgroup exchange (Agranoff *et al*, 1958; Paulus & Kennedy, 1960; Jungalwala, 1973). However, this process stimulates the labelling of a hormone-insensitive pool of inositol phospholipid (LaBarca *et al*, 1985). Pre-labelling cells with [^{3}H]inositol in the presence of $MnCl_2$ increased the incorporation of label into the phosphoinositides, and reduced free [^{3}H]inositol levels. However, unlike ACh pre-stimulation, subsequent acute AII stimulation resulted in a significant increase in the maximum phosphoinositide labelling, though the incremental increase in phosphoinositide and phosphoinositol labelling, and the loss of [^{3}H]inositol, were unaffected by $MnCl_2$ pre-treatment. This suggests that $MnCl_2$, unlike ACh, stimulated the labelling of a phosphoinositide pool distinct from that labelled by AII.

ACh pre-stimulation had no effect on the levels to which phosphoinositide labelling and free [^{3}H]inositol levels dropped in response to subsequent chronic (2 h) stimulation by AII. Furthermore, this treatment had no effect on the accompanying phosphoinositol response to AII (Figure 6.7). This suggests that the lipid synthesised in response to ACh pre-stimulation was accessible to the PLC activated in response to AII, and must therefore have been in the same hormone-sensitive pool. In contrast, when cells pre-labelled in the presence of $MnCl_2$ were subsequently stimulated for 2 h with AII, the pool of phosphoinositide labelled in response to $MnCl_2$ remained inaccessible to the AII-

stimulated PLC. Cells pre-treated with $MnCl_2$ showed the same incremental decrease in phosphoinositide labelling in response to chronic AII stimulation as did control cells, but showed a significantly reduced phosphoinositol response to AII. These data also suggest that, as in other cells, $MnCl_2$ stimulated the labelling of a hormone-insensitive pool of phosphoinositide.

It is possible that the hormone-insensitive pool of inositol phospholipid labelled in the presence of manganese represents labelling of 3-phosphorylated phospholipids. However, HPLC analysis of the phosphoinositol species formed in response to AII in bovine zfr cells indicates that 3-phosphate derivatives of the phosphoinositides are not formed (Bird *et al,* 1992d). Even if 3-phosphorylated phosphoinositides are not substrates for PLC activity (Fisher *et al,* 1992), FPLC and HPLC studies of the deacylated phosphoinositides from bovine zfr cells have also failed to detect multiple isomeric forms of phosphatidylinositol bisphosphate (Dr. I.M. Bird, personal communication).

In conclusion, the data presented in this chapter suggest that ACh and AII share a common mechanism of action in bovine zfr cells, and in addition stimulate the breakdown and resynthesis of a common hormone-sensitive pool of phosphoinositides which is distinct from the hormone-insensitive pool labelled by manganese. A similar situation appears to occur in rat adrenal zg cells stimulated with AII and vasopressin (Enyedi *et al,* 1988), and in the WRK-1 cell line stimulated with bradykinin and vasopressin (Monaco *et al,* 1990). The coupling of two or more receptors to a common pool of phosphoinositides in the same cell has important implications to the possible modulation of the effects of one agonist by another. Homologous desensitisation or sensitisation to either agonist must therefore occur at the levels of the receptor, whereas heterologous desensitisation would be expected to result from changes in the PLC-G_q protein complex.

CHAPTER 7

EFFECTS OF ACETYLCHOLINE AND ANGIOTENSIN II ON INTRACELLULAR CALCIUM CONCENTRATION IN INDIVIDUAL BOVINE ZFR CELLS

7.1 INTRODUCTION

It is now well established that intracellular Ca^{2+} plays a pivotal role in the steroidogenic actions of AII and ACh in adrenocortical zg cells. Studies of $^{45}Ca^{2+}$ efflux from superfused rat zg cells, and measurement of $[Ca^{2+}]_i$ in aequorin-loaded rat zg cells have shown that AII stimulates a rapid and sustained rise in $[Ca^{2+}]_i$, with a dose-dependency correlating well with the aldosterone-stimulating action of the peptide (Balla *et al*, 1985a; Braley *et al*, 1984,1986). Similar results have been obtained in zg cells from bovine adrenal cortex (Kojima *et al*, 1985a,b,d). The initial sharp increase in $[Ca^{2+}]_i$ in response to AII occurs through the $Ins(1,4,5)P_3$-mediated release of Ca^{2+} from intracellular stores (Barret *et al*, 1986a; Gupta *et al*, 1992; Kojima *et al*, 1984; Balla *et al*, 1989), while the sustained phase of the AII $[Ca^{2+}]_i$ response is maintained through influx of extracellular Ca^{2+} (Capponi *et al*, 1984). The importance of this Ca^{2+} influx is evident from the inhibitory effects of Ca^{2+} channel blockers such as lanthanum on AII-stimulated aldosterone secretion (Fakunding & Catt, 1980), and recent studies have proposed a dominant role of non-dihydropyridine-sensitive Ca^{2+} channels in AII-stimulated Ca^{2+} influx in bovine zg cells (Ambroz & Catt, 1992). Although less study has been made of ACh effects on zg cell $[Ca^{2+}]_i$, it is clear that ACh does promote a rapid elevation of $[Ca^{2+}]_i$ in aquorin-loaded bovine zg cells (Kojima *et al*, 1986a).

The effects of AII and ACh on $[Ca^{2+}]_i$ in zfr cells has received comparatively less attention. That AII has no effect on $^{45}Ca^{2+}$ efflux or $[Ca^{2+}]_i$ in rat zfr cells (Williams *et al*, 1981; Braley *et al*, 1986) is in agreement with the reported lack of effect of the peptide on rat zfr cell steroidogenesis (Whitley *et al*, 1982). By contrast, both AII and ACh stimulate rapid dose-dependent increases in $[Ca^{2+}]_i$ in populations of fura-2 loaded bovine zfr cells (Walker *et al*, 1990,1991), and it has been proposed that in this preparation, AII and ACh mobilise a common intracellular pool of Ca^{2+} (Walker *et al*, 1991), despite

evidence suggesting the existence of at least three distinct Ca^{2+} pools in bovine adrenal cortex (Guillemette *et al*, 1991).

The measurement of $[Ca^{2+}]_i$ in cell populations (as in the above studies) produces a population average Ca^{2+} signal, and thus provides no information regarding the $[Ca^{2+}]_i$ responses of individual cells. It is known that in individual rat and bovine zg cells, AII stimulates oscillations in $[Ca^{2+}]_i$ (Johnson *et al*, 1989; Quinn *et al*, 1988). That the frequency of such oscillations was dependent on the concentration of AII used raises the possibility that in zg cells, aldosterone secretion may be regulated by a Ca^{2+} signal that is frequency, rather than amplitude, encoded. Since no information is available regarding the effects of AII or ACh on $[Ca^{2+}]_i$ in individual zfr cells from any species, the experiments described in this chapter were performed in order to investigate the $[Ca^{2+}]_i$ responses of single fura-2 loaded bovine zfr cells to these agonists.

7.2 RESULTS

7.2.1 Cellular loading and calibration of fura-2

In order to investigate the kinetics and concentration dependence of fura-2 uptake into bovine zfr cells, cultured cells were exposed to fura-2 AM under various experimental conditions, and the internalised dye quantified by spectrofluorimetry. Following thorough washing of loaded cells, cells were sonicated to release intracellular dye, and the fluorescence intensity emitted on excitation at 362 nm recorded. This wavelength represents the isosbestic point of fura-2 (Figure 2.1), and therefore fluorescence measurements made at 362 nm are proportional to the absolute amount of dye present, and unaffected by any changes in free Ca^{2+} concentration.

As shown in Figure 7.1, fura-2 uptake was concentration dependent. Uptake increased linearly over the concentration range 1 - 10 μM, reaching a plateau at higher dye concentrations. A time-dependent increase in fura-2 uptake was also observed (Figure 7.2). Uptake was maximal following 60 min exposure to the dye, and this maximal level of uptake was maintained at loading times of at least 2 h.

In order to relate changes in the fluorescence ratio F_{340}/F_{380} obtained from fura-2 loaded cells to changes in intracellular Ca^{2+} concentration, the fluorescence ratios obtained from solutions of known free Ca^{2+} concentration in the presence of 0.1 μM fura-2 free acid were recorded, and used to construct a calibration curve (Figure 7.3). The experimental conditions under which measurements were made from standard solutions were designed to mimic as closely as possible measurements made from cells: the magnification, and the aperture size through which emitted light was recorded were the same for cell and calibration measurements. Furthermore, the absolute intensity of light emitted at the two excitation wavelengths was of a similar order of magnitude for both cell and calibration measurements (ca. 10^5 photons/sec). As shown in Figure 7.3, a linear relationship existed between free Ca^{2+} concentration (50-800 nM) and the fluorescence ratio F_{340}/F_{380}. The ratios obtained were relatively constant between different batches of standards up to 400 nM free Ca^{2+}. However, larger variation in ratios was encountered both between and within separate batches at free Ca^{2+} concentrations of 600-800 nM, reflecting the upper limit of sensitivity of fura-2, which becomes saturated at Ca^{2+} concentrations approaching 1.0 μM (Grynkiewicz *et al.* 1985).

FIGURE 7.1

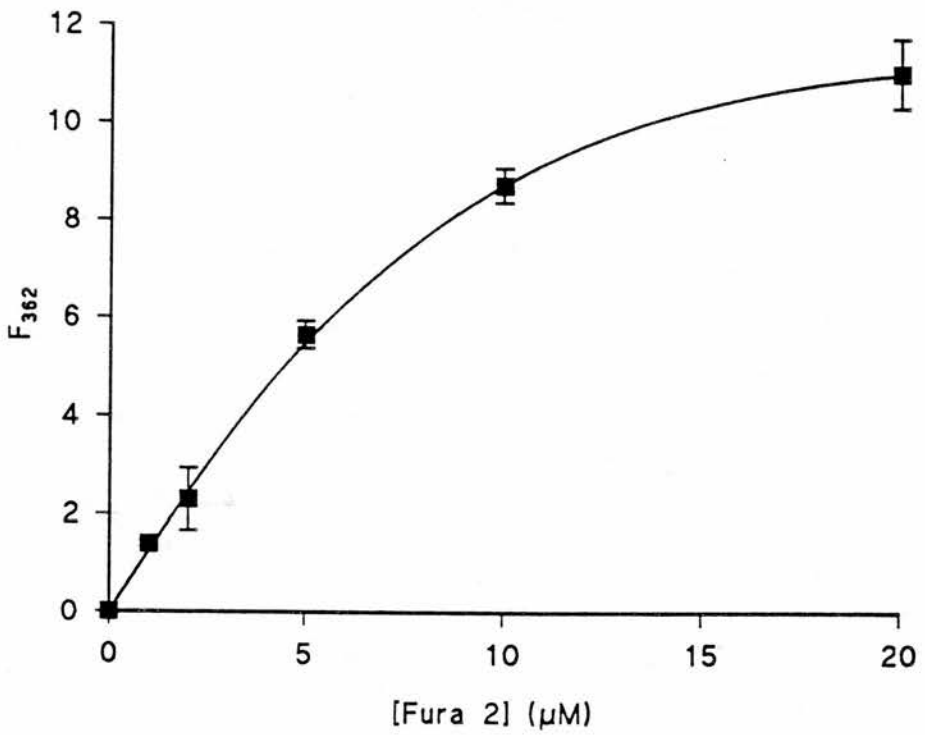

FIGURE 7.1 : Concentration dependence of fura-2 uptake into bovine zfr cells in monolayer culture. Cells were incubated with fura-2 at the concentrations indicated for 60 min. Following extensive washing, cells were scraped from the culture plastic and sonicated to release intracellular dye. The fluorescence signal was recorded at 510 nm with an excitation wavelength of 362 nm. Values are corrected for autofluorescence recorded from cells loaded with vehicle only, and represent the mean ± SD of triplicate determinations from a representative experiment. Similar results were obtained in a further two experiments.

FIGURE 7.2

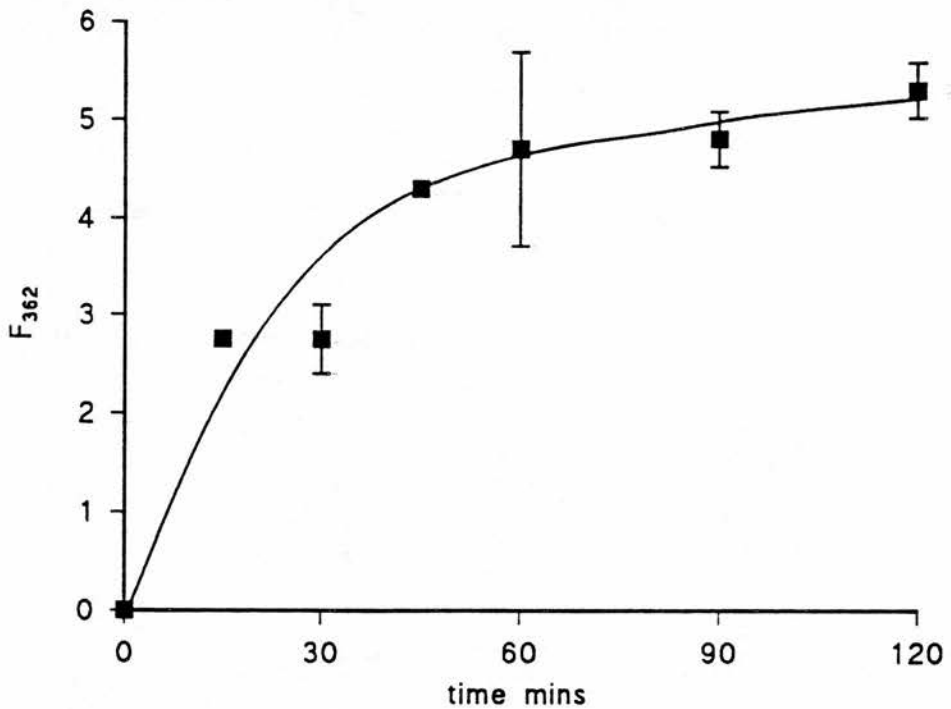

FIGURE 7.2 : Time course of fura-2 uptake by cultured bovine zfr cells. Cells were loaded with fura-2 (10 μM) for the times indicated and processed as in Figure 7.1. Values represent the mean ± SD of triplicate determinations from a representative experiment. Similar results were obtained in a second experiment.

FIGURE 7.3

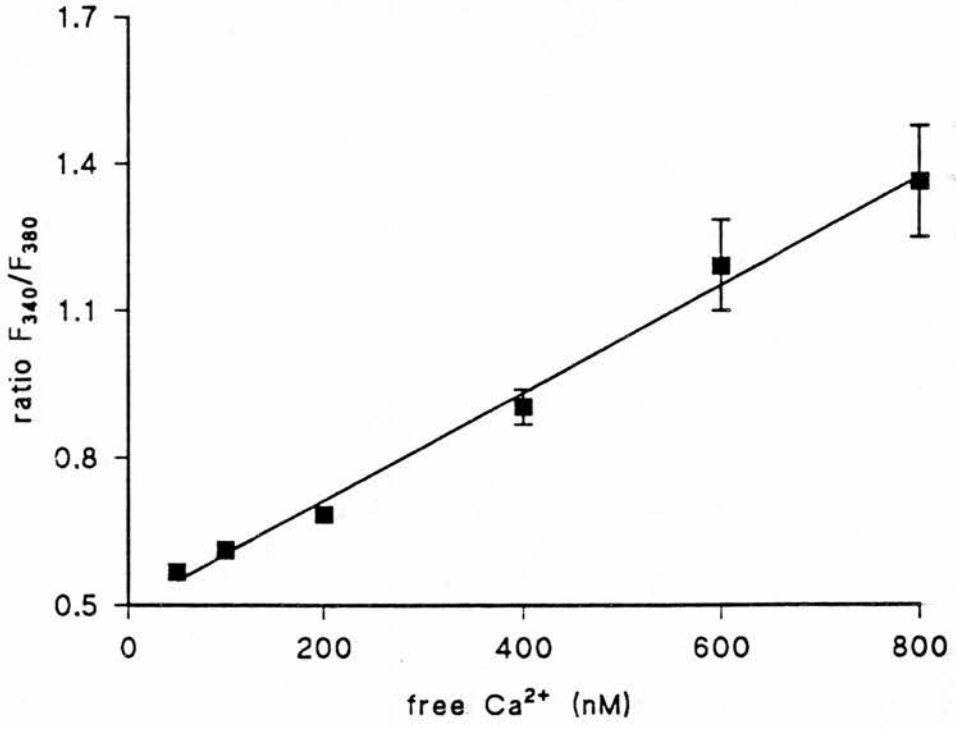

FIGURE 7.3 : *In vitro* calibration showing the relationship between the fluorescence ratio F_{340}/F_{380} for fura-2 as a function of free Ca^{2+} concentration. Measurements were carried out at 37°C through the optics of a Nikon Diaphot inverted microscope using 500 µl of Kreb's buffer of defined free Ca^{2+} concentration containing fura-2 free acid (0.1 µM) Data points are the mean ± s.e.m. of triplicate determinations from three sets of standard solutions prepared on different occasions. The correlation coefficient of regression was 0.997.

7.2.2 Effect of acetylcholine on intracellular Ca^{2+} concentration in single bovine zfr cells.

Cells were loaded with fura-2 (10 μM) for 1 - 2 h at 37°C. Following removal of extracellular fura-2, fluorescence measurements were made from individual zfr cells as described in section 2.6.2. ACh (10^{-6} - 10^{-4}M) was presented to the cells as a continuous perfusion. A total of 38 cells from at least eight separate cell preparations were analysed in this way. Representative experiments are shown in Figures 7.4 - 7.8.

Mean resting F_{340}/F_{380} ratio was 0.57, corresponding to an intracellular free Ca^{2+} concentration of 69 nM. This value is very close to the resting $[Ca^{2+}]_i$ previously determined in single fura-2 loaded bovine zg cells (61.2 nM, Johnson *et al,* (1989)). On stimulation with ACh, 82% of cells responded with an increase in $[Ca^{2+}]_i$. All cells which responded to ACh showed an initial sharp increase in $[Ca^{2+}]_i$. Although the magnitude of this initial response was extremely variable between cells, a dose-dependent trend was evident. At ACh concentrations of 10^{-6}, 10^{-5} and 10^{-4}M, the mean initial peak $[Ca^{2+}]_i$ levels were 370 nM (range 233 - 589 nM), 470 nM (range 188 - 1077 nM) and 613 nM (range 461 - 734 nM) respectively. Following this initial increase in $[Ca^{2+}]_i$, the characteristics of the fluorescence signal were variable, with cells showing either oscillatory or non-oscillatory responses. Considering firstly those cells which showed non-oscillatory responses, the nature of the Ca^{2+} signal again appeared to be dose-dependent. At 10^{-6}M ACh, the fluorescence ratio decayed exponentially, returning to the original resting value within 5 - 10 min (Figure 7.4). At 10^{-5} and 10^{-4}M ACh, the fluorescence signal decayed to a new elevated level which was sustained for several minutes (Figures 7.5 and 7.6 respectively). The mean plateau $[Ca^{2+}]_i$ was 231 nM at 10^{-5} M ACh and 252 nM at 10^{-4}M Ach. 100% of cells stimulated with 10^{-6}M ACh behaved in this way, while 60% of cells stimulated with 10^{-5}M ACh, and 75% of cells stimulated with 10^{-4}M ACh showed this pattern of responsiveness.

The remaining 40% and 25% of cells stimulated with 10^{-5} and 10^{-4}M ACh showed oscillatory responses following the initial sharp increase in $[Ca^{2+}]_i$. No oscillations in $[Ca^{2+}]_i$ were observed in response to 10^{-6}M ACh. When stimulated with 10^{-5}M ACh, however, following the initial sharp increase in $[Ca^{2+}]_i$, the level of Ca^{2+} decreased to basal levels with sharp oscillations in $[Ca^{2+}]_i$ superimposed over the falling signal (Figure 7.7). $[[Ca^{2+}]_i$ then oscillated between basal and stimulated levels for up to 10 min. The average amplitude of the individual spikes was 436 nM (range 188 - 734 nM), and the average period of the oscillations was 32.6 sec. At 10^{-4}M ACh, oscillations in $[Ca^{2+}]_i$

FIGURE 7.4

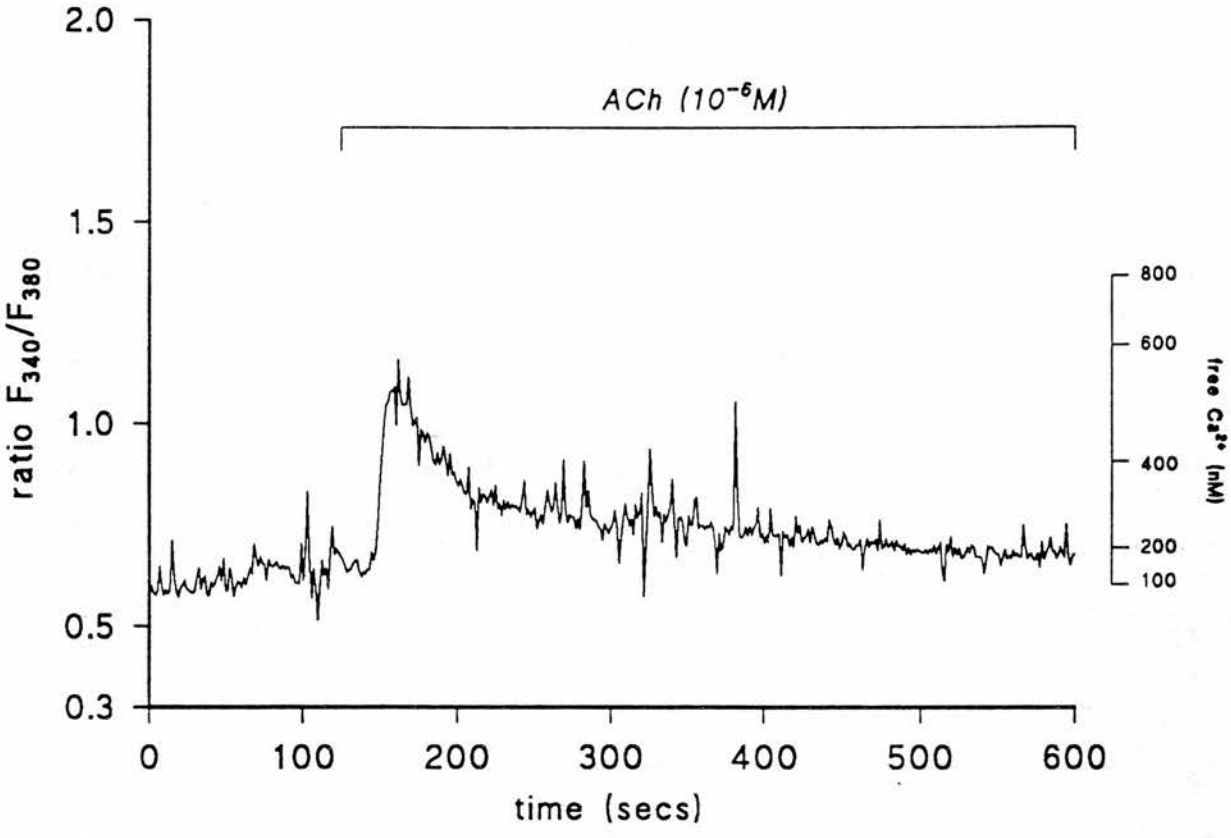

FIGURE 7.4 : Effect of acetylcholine (10^{-6}M) on the intracellular Ca^{2+} concentration of a single fura-2 loaded bovine zfr cell. Acetylcholine was delivered as a continuous perfusion, indicated by the horizontal bar. The change in fluorescence ratio F_{340}/F_{380} over time is indicated on the left vertical axis, and the corresponding free Ca^{2+} concentration is indicated on the right.

FIGURE 7.5

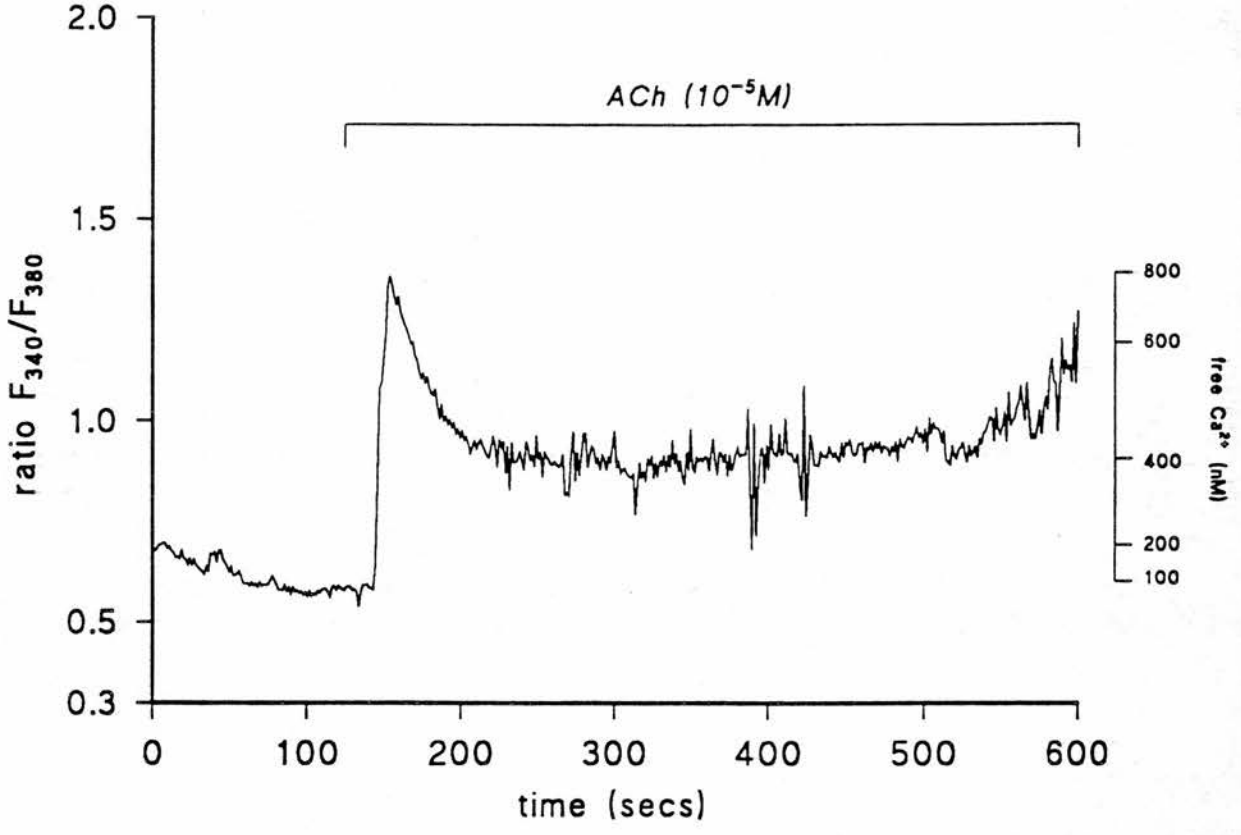

FIGURE 7.5 : Effect of acetylcholine (10^{-5}M) on the intracellular Ca^{2+} concentration of a single fura-2 loaded bovine zfr cell. Acetylcholine was delivered as a continuous perfusion, indicated by the horizontal bar. The change in fluorescence ratio F_{340}/F_{380} over time is indicated on the left vertical axis, and the corresponding free Ca^{2+} concentration is indicated on the right.

FIGURE 7.6

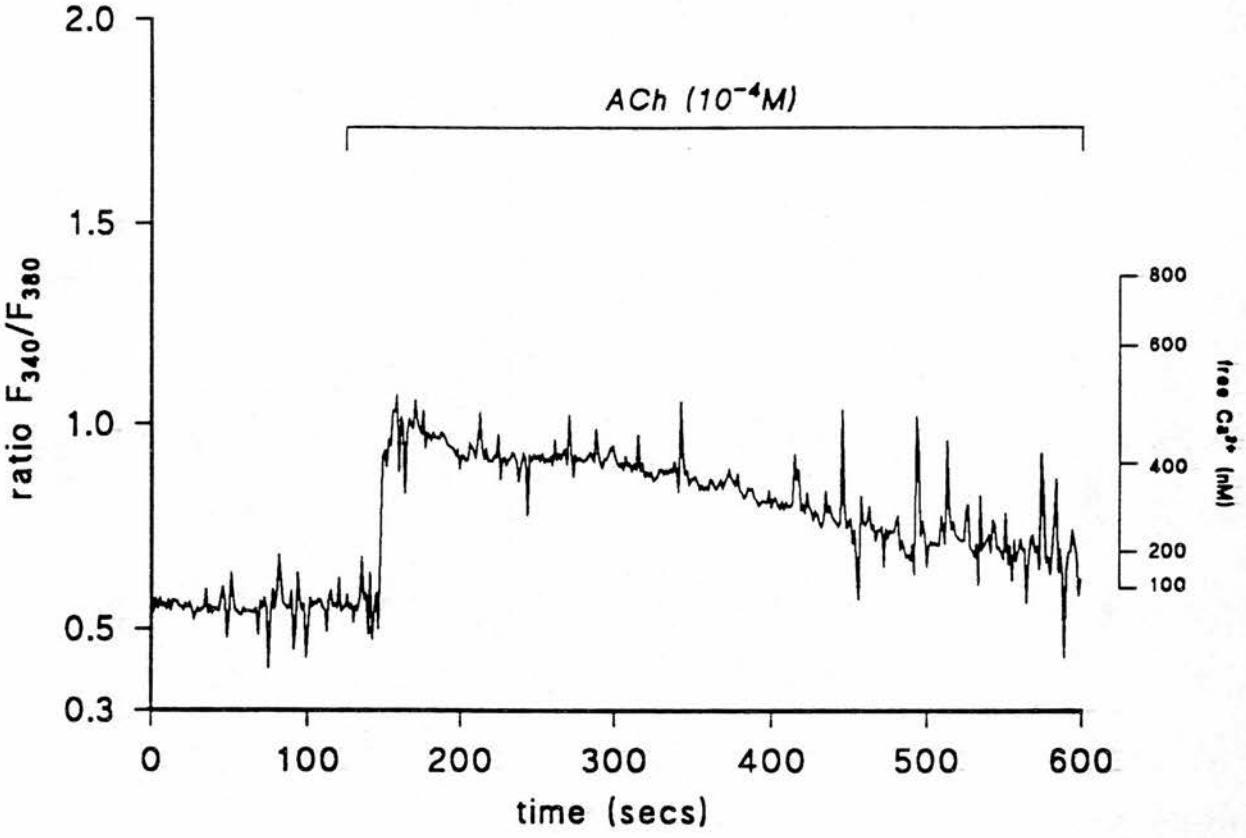

FIGURE 7.6 : Effect of acetylcholine (10^{-4}M) on the intracellular Ca^{2+} concentration of a single fura-2 loaded bovine zfr cell. Acetylcholine was delivered as a continuous perfusion, indicated by the horizontal bar. The change in fluorescence ratio F_{340}/F_{380} over time is indicated on the left vertical axis, and the corresponding free Ca^{2+} concentration is indicated on the right.

FIGURE 7.7

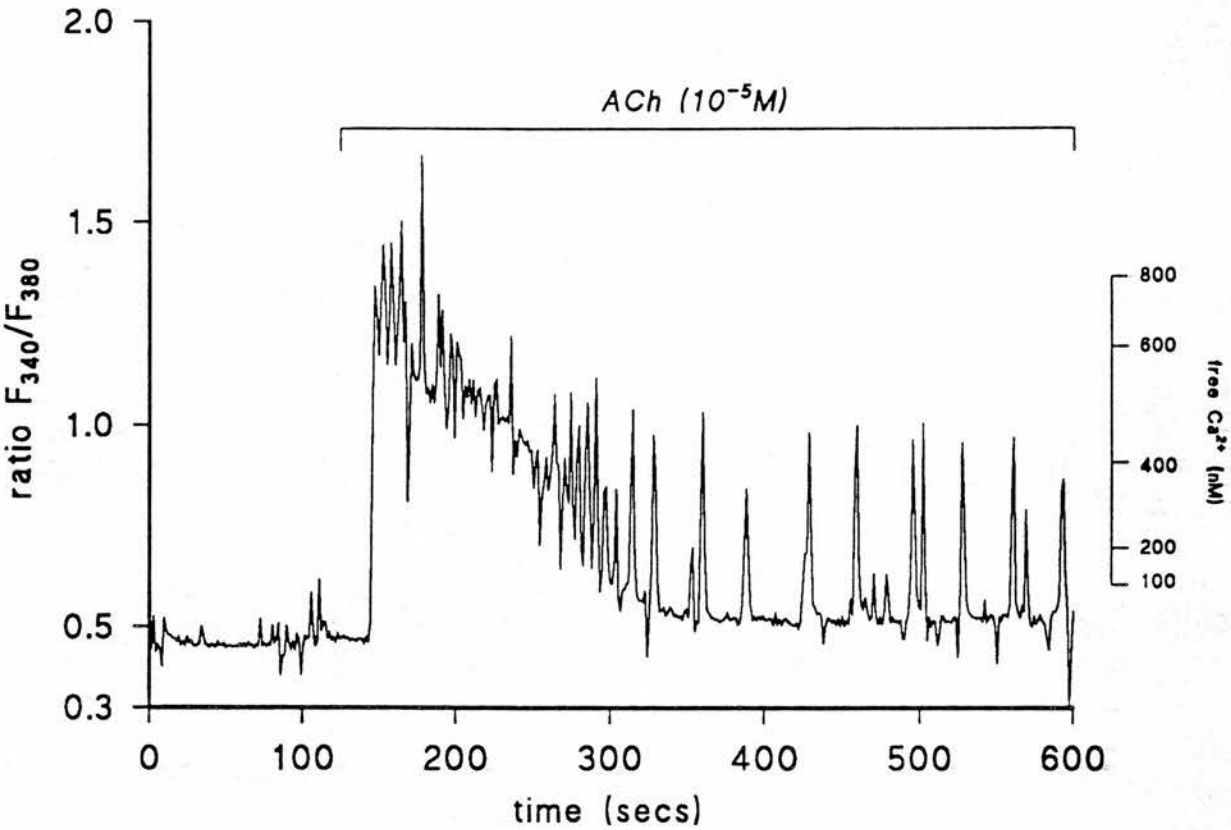

FIGURE 7.7 : Effect of acetylcholine (10^{-5}M) on the intracellular Ca^{2+} concentration of a single fura-2 loaded bovine zfr cell. Acetylcholine was delivered as a continuous perfusion, indicated by the horizontal bar. The change in fluorescence ratio F_{340}/F_{380} over time is indicated on the left vertical axis, and the corresponding free Ca^{2+} concentration is indicated on the right.

FIGURE 7.8

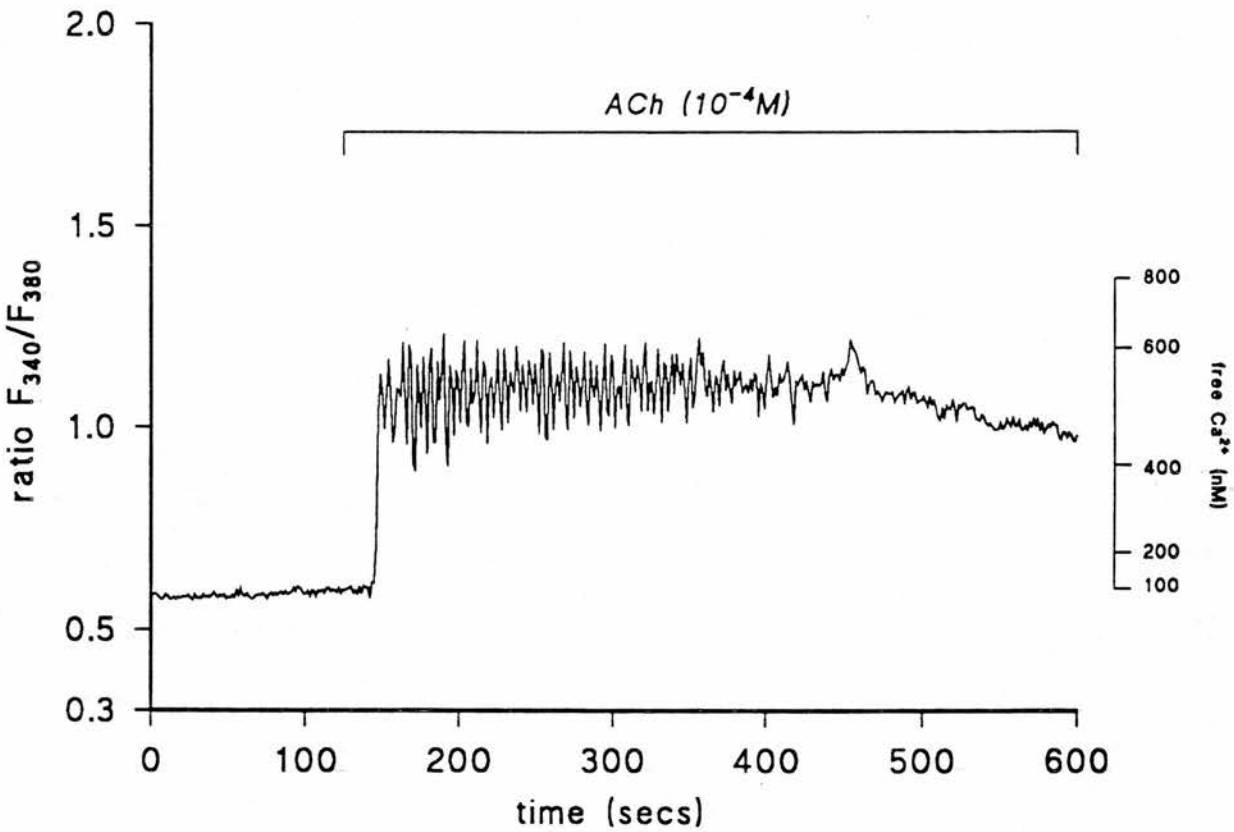

FIGURE 7.8 : Effect of acetylcholine (10^{-4}M) on the intracellular Ca^{2+} concentration of a single fura-2 loaded bovine zfr cell. Acetylcholine was delivered as a continuous perfusion, indicated by the horizontal bar. The change in fluorescence ratio F_{340}/F_{380} over time is indicated on the left vertical axis, and the corresponding free Ca^{2+} concentration is indicated on the right.

FIGURE 7.9

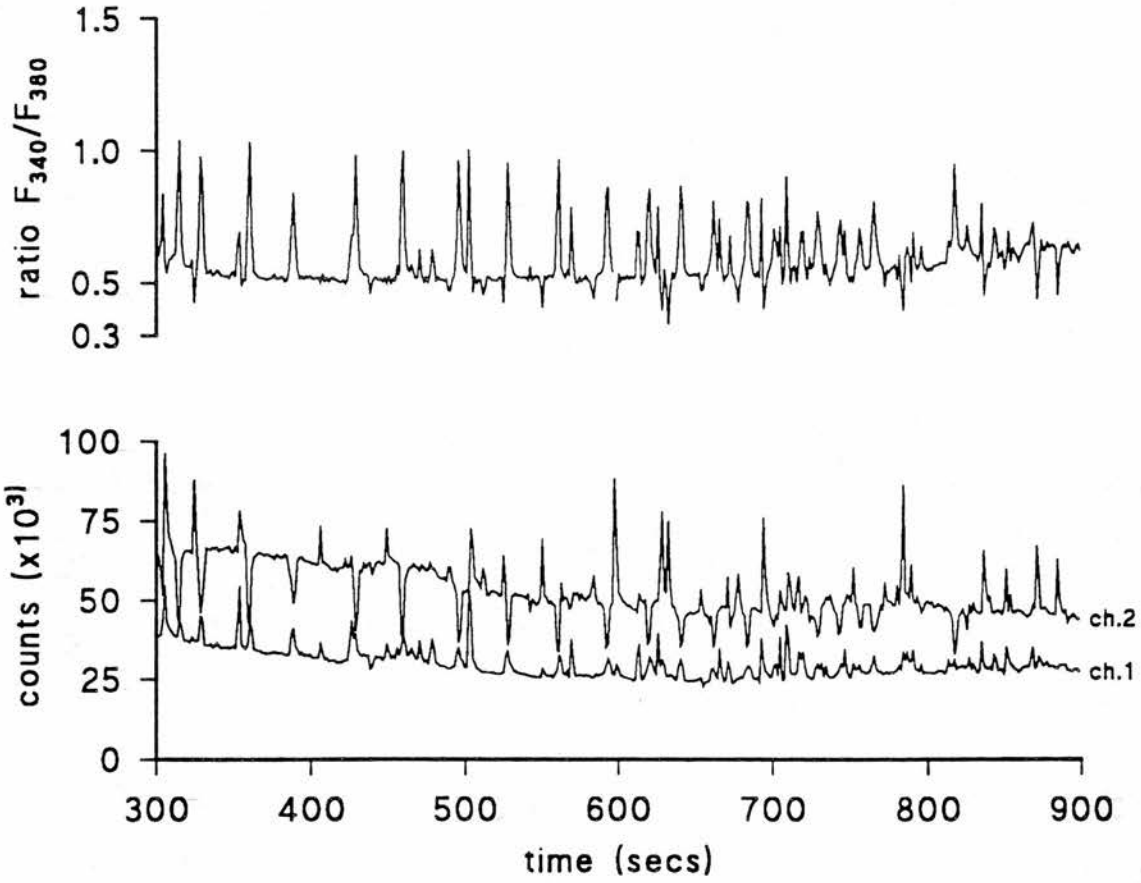

FIGURE 7.9 : Acetylcholine (10^{-5}M)-stimulated oscillations in $[Ca^{2+}]_i$ (upper panel), and the corresponding fluorescence intensities emitted by the cell when excited alternately at 340 nm (ch.2) and 380 nm (ch.1).

FIGURE 7.10

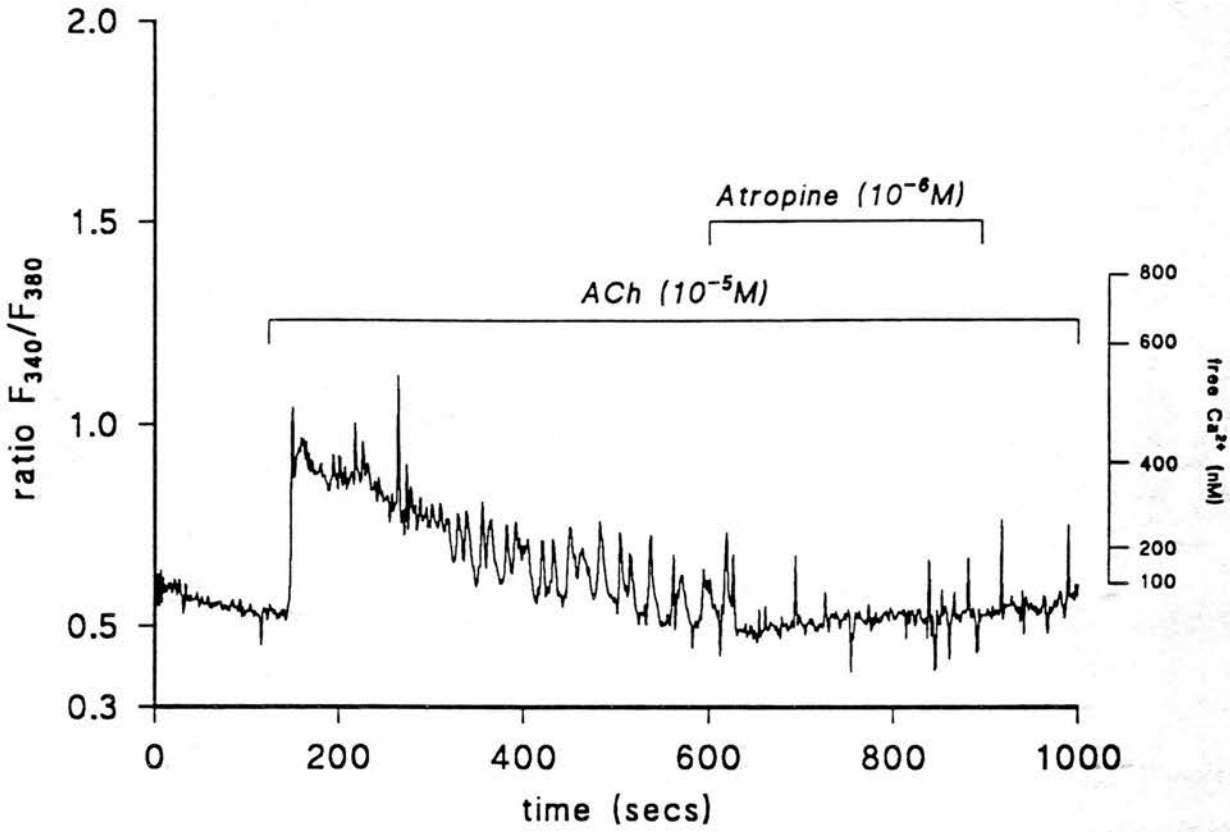

FIGURE 7.10 : Effect of atropine (10^{-6}M) on acetylcholine- induced oscillations in $[Ca^{2+}]_i$ of a single fura-2 loaded bovine zfr cell. Test substances were delivered as continuous perfusions, indicated by the horizontal bars. The change in fluorescence ratio F_{340}/F_{380} over time is indicated on the left vertical axis, and the corresponding free Ca^{2+} concentration is indicated on the right.

were of very high frequency, oscillating around an elevated level of $[Ca^{2+}]_i$, before beginning to decay (Figure 7.8).

These oscillations in $[Ca^{2+}]_i$ could be distinguished from random spikes due to electrical noise which occasionally occurred during experiments by analysis of the raw photon counts at 340 and 380 nm excitation wavelength (Figure 7.9). The traces for each excitation wavelength are in theory perfectly antiparallel. It can be seen from Figure 7.9 that true transient increases in $[Ca^{2+}]_i$ were associated with antiparallel peaks in the two traces, while for random noise spikes the peaks in each channel were in the same (upwards) direction.

The effects of the (non-selective) muscarinic antagonist atropine on ACh-induced Ca^{2+} oscillations are shown in Figure 7.10. A cell in which $[Ca^{2+}]_i$ was oscillating in response to ACh (10^{-5}M) was then co-perfused with atropine (10^{-6}M). Oscillations ceased almost immediately in the presence of atropine. A similar result was obtained in a second experiment in which the effect of atropine was investigated

7.2.3 Effect of angiotensin II on intracellular Ca^{2+} concentration in single bovine zfr cells.

In a further series of experiments cells treated with fura-2 as above were stimulated by continuous perfusion of AII (10^{-9} - 10^{-7}M). A total of 27 individual cells from five separate experiments were studied. Representative experiments are shown in Figures 7.11 - 7.13. In contrast to the $[Ca^{2+}]_i$ signals observed in response to ACh, those $[Ca^{2+}]_i$ responses to AII were relatively homogeneous, and no oscillations in $[Ca^{2+}]_i$ were observed at any concentration of AII. 76% of cells responded to AII with an increase in $[Ca^{2+}]_i$. As was observed in response to ACh, the Ca^{2+} signal showed an initial rapid rise in response to AII. The mean peak $[Ca^{2+}]_i$ values in response to 10^{-9}, 10^{-8} and 10^{-7}M AII were 313 nM (range 192 - 825 nM), 408 nM (range 208 - 734 nM) and 387 nM (range 192 - 1293 nM) respectively. The subsequent changes in $[Ca^{2+}]_i$ in response to AII appeared to be dose-related. At 10^{-9}M, the peak $[Ca^{2+}]_i$ invariably decayed rapidly, reaching resting levels within 1 - 2 min (Figure 7.11). At 10^{-8}M AII, the Ca^{2+} signal again decayed exponentially to resting levels. but with a longer half-time (resting levels were reached within 4 - 6 min). When stimulated with 10^{-7}M AII, the initial peak $[Ca^{2+}]_i$ signal decayed exponentially before settling at a new elevated level (mean plateau level 183 nM) which was maintained for at least 5 min.

FIGURE 7.11

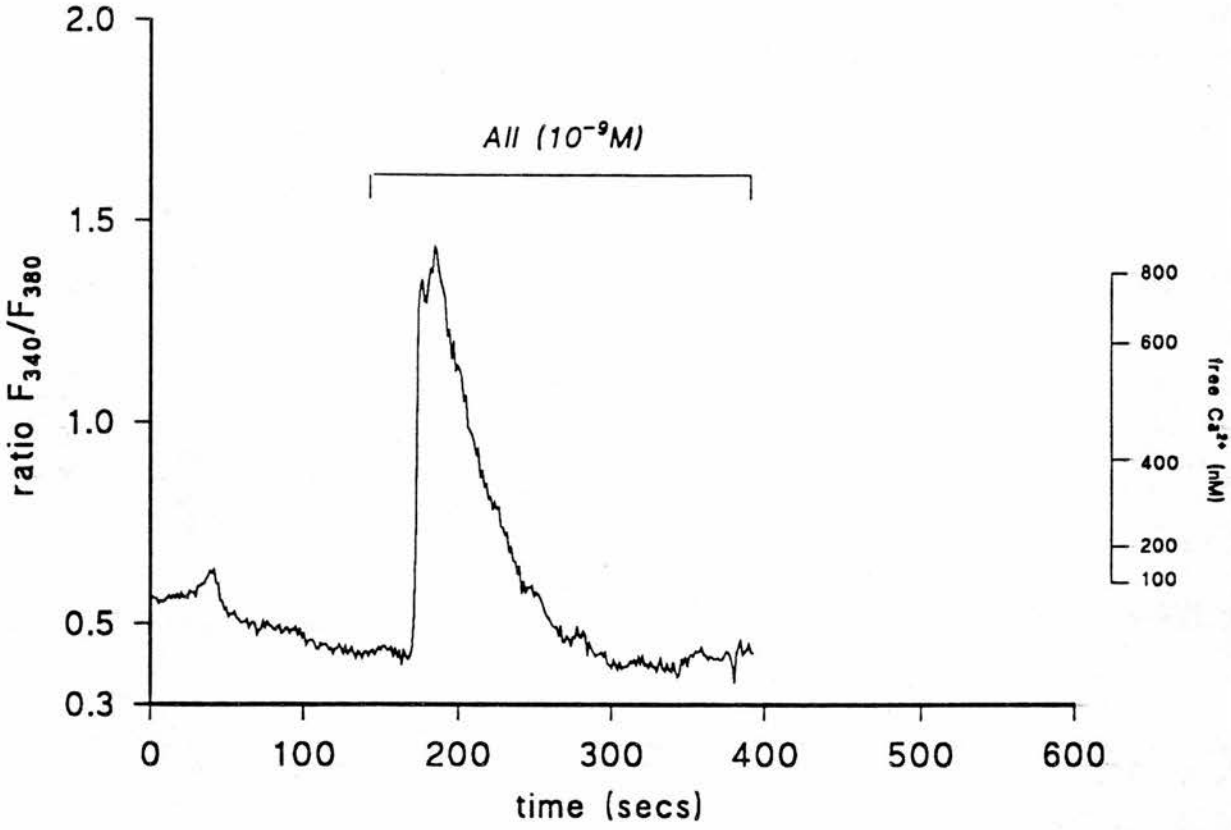

FIGURE 7.11 : Effect of angiotensin II (10^{-9}M) on the intracellular Ca^{2+} concentration of a single fura-2 loaded bovine zfr cell. Angiotensin II was delivered as a continuous perfusion, indicated by the horizontal bar. The change in fluorescence ratio F_{340}/F_{380} over time is indicated on the left vertical axis, and the corresponding free Ca^{2+} concentration is indicated on the right.

FIGURE 7.12

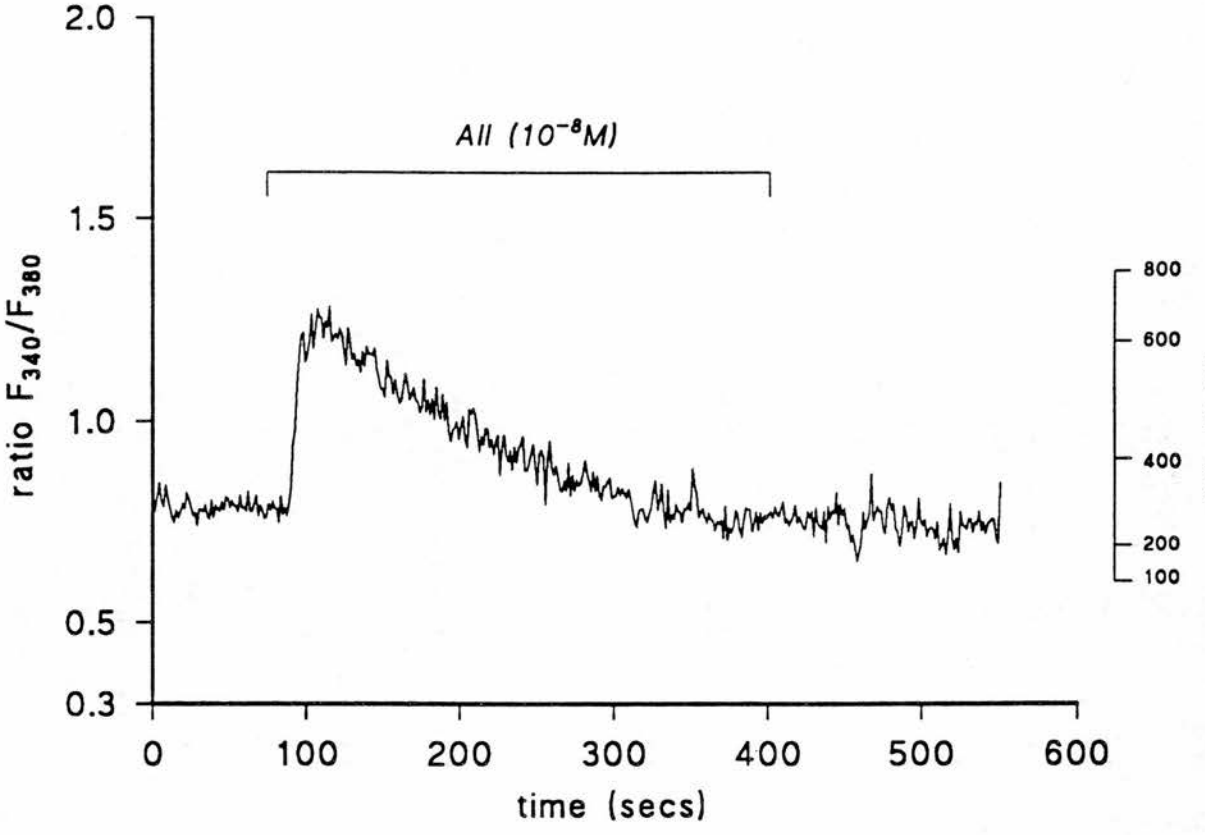

FIGURE 7.12 : Effect of angiotensin II (10^{-8}M) on the intracellular Ca^{2+} concentration of a single fura-2 loaded bovine zfr cell. Angiotensin II was delivered as a continuous perfusion, indicated by the horizontal bar. The change in fluorescence ratio F_{340}/F_{380} over time is indicated on the left vertical axis, and the corresponding free Ca^{2+} concentration is indicated on the right.

FIGURE 7.13

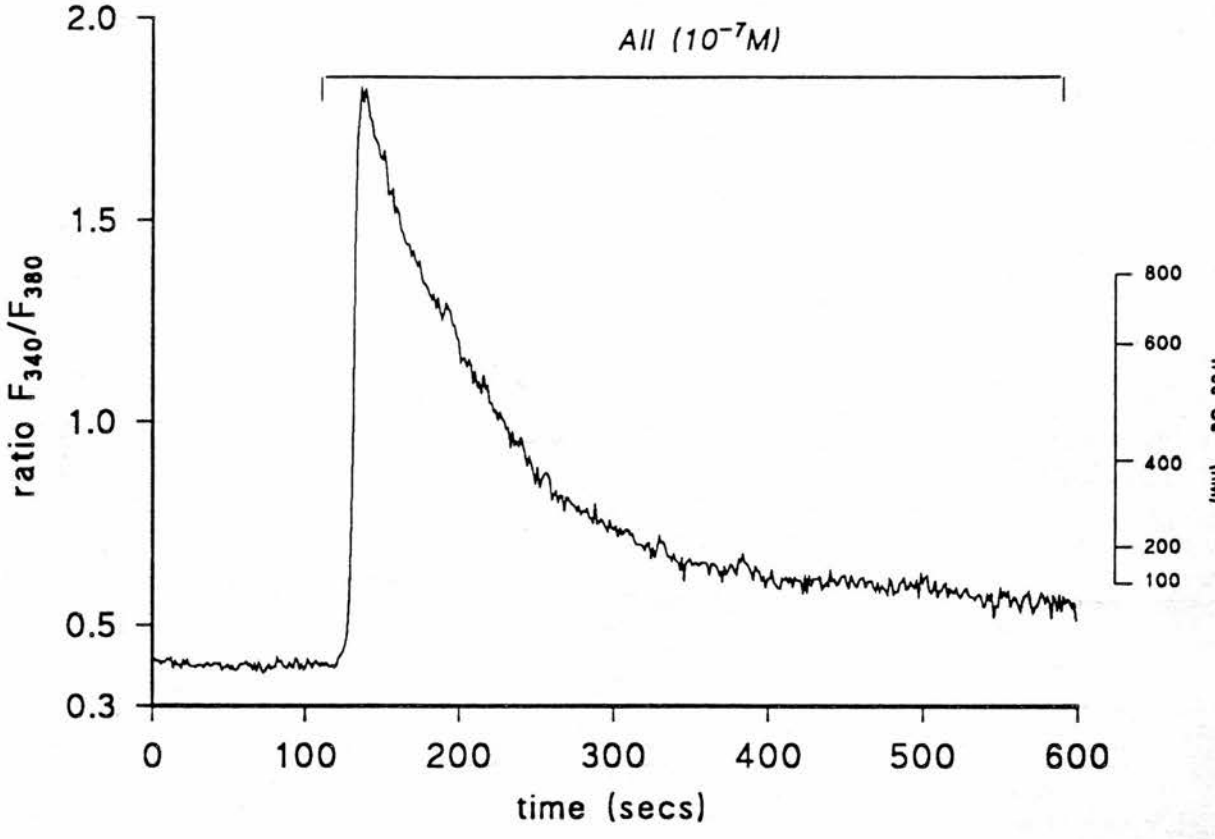

FIGURE 7.13 : Effect of angiotensin II (10^{-7}M) on the intracellular Ca^{2+} concentration of a single fura-2 loaded bovine zfr cell. Angiotensin II was delivered as a continuous perfusion, indicated by the horizontal bar. The change in fluorescence ratio F_{340}/F_{380} over time is indicated on the left vertical axis, and the corresponding free Ca^{2+} concentration is indicated on the right.

7.3 DISCUSSION

Fura-2 has now become the most widely used indicator of $[Ca^{2+}]_i$. The use of this indicator, combined with dual excitation fluorescence microscopy and digital imaging techniques has revealed complex spatio-temporal Ca^{2+} responses in single cells from many tissues/species not apparent from population studies (reviewed in Berridge & Irvine, 1989; Fewtrell, 1993). While oscillatory $[Ca^{2+}]_i$ behaviour has been reported in single rat (Quinn *et al*, 1988) and bovine (Johnson *et al*, 1989) adrenocortical zg cells stimulated with AII, the $[Ca^{2+}]_i$ responses of single zfr cells to AII have not been studied. Neither have the effects of ACh on $[Ca^{2+}]_i$ in single adrenocortical cells been investigated. The present results extend previous studies of AII and ACh effects on $[Ca^{2+}]_i$ in bovine zfr cells populations (Walker *et al*, 1990,1991), and reveal a heterogeneity of response of individual cells to these agonists.

Despite its widespread use, there are a number of problems associated with the loading and calibration of $[Ca^{2+}]_i$ using fura-2. Different cell types differ in their ability to take up and de-esterify fura-2 AM (Malgaroli *et al*, 1988), and it was necessary therefore to optimise the conditions under which the dye is loaded. As shown in figures 7.1 and 7.2, fura-2 uptake was both time and concentration dependent. The loading conditions chosen (10 μM, 1-2 h) were sufficient to allow a high signal:noise ratio, while presumably maintaining relatively low intracellular concentrations of the dye. This is desirable since at high fura-2 concentrations, buffering of intracellular Ca^{2+} can occur (Moore *et al*, 1990). Although the absolute concentration of fura-2 was not known, that adequate levels of loading were achieved at micromolar concentrations of the dye, together with the known sensitivity of fura-2 (Grynkiewicz *et al*, 1985), suggest that buffering of $[Ca^{2+}]_i$ did not occur. This is further supported by the relatively low $[Ca^{2+}]_i$ measured under resting conditions (69 nM).

A further problem associated with the use of fura-2 is the method of calibration chosen to relate ratio measurements to intracellular free Ca^{2+} concentrations. Two approaches are in widespread use, the *in vitro* and *in situ* methods, which may produce significantly different results (Almers & Neher, 1985; Tsein *et al*, 1985). The *in situ* method involves equilibrating cells with buffers of known free Ca^{2+} concentration in the presence of an ionophore, and recording the ratios obtained from such cells. The free $[Ca^{2+}]_i$ is calculated according to Grynkiewicz et al (1985) following estimation of the minimum and maximum fluorescence ratios measured at zero and saturating concentrations of Ca^{2+} (R_{min}, R_{max}). This method suffers from the problem that ionphores may not fully equilibrate intra- and extracellular Ca^{2+}, and in addition, fura-2 may leak from ionophore-

treated cells (Roe *et al*, 1990). Furthermore, R_{max} must be measured in lysed cells, leading to loss of intracellular fura-2. This makes the *in situ* method unsuitable for use in single cell measurements, since the dye escapes from the microscope field of view, precluding accurate measurement of R_{max}. While these problems are overcome by the *in vitro* method utilised here, ratio measurements made from buffered solutions may not truly reflect ratios made from fura-2 loaded cells since the intracellular milieu obviously differs from experimental solutions in terms of viscosity, local ionic strength and polarity, all of which are known to affect fura-2 ratio measurements (Roe *et al*, 1990; Poenie, 1990).

Given these considerations, the *in vitro* method of calibration was used in the present study (Figure 7.3). Ratio measurements made from standard Ca^{2+}-buffered solutions were both internally and externally consistent at free Ca^{2+} concentrations up to 400 nM. Above this concentration, measurements were subject to much greater variability. This variability stems from the gradual saturation of fura-2 at free Ca^{2+} concentrations approaching 1 μM (Grynkiewicz *et al*, 1985). This is apparent from both Figure 7.3 and from the Ca^{2+} titration curve presented in Figure 2.1. This reduced sensitivity of fura-2 to changes in high $[Ca^{2+}]$ inevitably leads to increased error both in the preparation of buffered solutions of free $[Ca^{2+}]$ above 400-500 nM, and in the ratio measurements made from such solutions. For these reasons, both ratio measurements and estimated free Ca^{2+} concentrations are presented in the individual traces shown in Figures 7.4 - 7.13, and the errors associated with measurement of high free $[Ca^{2+}]$ must be considered.

The mean resting $[Ca^{2+}]_i$ of individual bovine zfr cells was 69 nM. This value is close to that previously reported in single bovine (61 nM) and rat (82 nM) zg cells (Johnson *et al*, 1989; Quinn *et al*, 1988), and also correlates well with that determined in populations of bovine zfr cells (75 nM, Walker *et al*, 1991). However, this resting value is significantly lower than that determined in populations of aquorin-loaded bovine (136 nM) and rat (ca. 240 nM) zg cells (Kojima *et al*, 1986a; Braley *et al*, 1986). Several factors may contribute to this discrepancy including, for example, the use of different Ca^{2+} indicators between studies. Quantitative differences in $[Ca^{2+}]_i$ measured by fura-2 and aquorin have been attributed to the Ca^{2+}-binding properties of the two dyes (Quinn *et al*, 1988). Fura-2 is a linear indicator of $[Ca^{2+}]_i$ (Grynkiewicz *et al*, 1985), whereas the fluorescence signal emitted by aquorin is a function of the cube of the Ca^{2+} concentration. A further possibility is that spontaneous oscillations in resting $[Ca^{2+}]_i$ contribute to the population average $[Ca^{2+}]_i$. Johnson *et al* (1989), observing spontaneous Ca^{2+} oscillations in individual bovine zg cells, suggested that this factor must have resulted in over-estimation of resting $[Ca^{2+}]_i$ in zg cell populations. Spontaneous oscillations in the

resting $[Ca^{2+}]_i$ were not observed in bovine zfr cells in the present study, and it is likely that this stability in zfr cell resting $[Ca^{2+}]_i$ explains the good agreement between single cell and population determinations of resting $[Ca^{2+}]_i$ in bovine zfr cells (69 nM *vs* 75 nM) compared with bovine zg cells (61 nM *vs* 136 nM).

When stimulated with ACh, bovine zfr cells showed a heterogeneous pattern of responsiveness. Following an initial rapid increase in $[Ca^{2+}]_i$ observed in all cells which responded to ACh, cells could be divided into those that showed agonist-induced oscillations in $[Ca^{2+}]_i$, and those that did not. It is unlikely that this heterogeneity of response can be explained by the presence of contaminating cell types. The Sephadex column filtration method for zfr cell purification employed in this study is known to produce an extremely pure cell preparation from which secretion of aldosterone and androstenedione is undetectable (Williams *et al*, 1989). Furthermore, a similar heterogeneity of $[Ca^{2+}]_i$ response has been observed in single cells from a homogeneous population of clonal pancreatic insulinoma cells stimulated with a single agonist (Prentki *et al*, 1988). The reasons underlying this heterogeneity of responsiveness are at present unknown. It cannot be explained by differences in the cell cycle between test cells since heterogeneous responses were also observed between individual daughter cells from a smooth muscle cell line immediately on completion of cell division (Ambler *et al*, 1988). An interesting feature of this cell-specific heterogeneity of response is that insulinoma cells responded in exactly the same way when restimulated with agonist. This phenomena was termed the Ca^{2+} fingerprint of the cell (Prentki *et al*, 1988), and has also been observed in vasopressin-stimulated hepatocytes (Rooney *et al*, 1989). It is unknown whether bovine zfr cells show a similar Ca^{2+} fingerprint since cells were completely unresponsive to subsequent further challenge, even at high agonist concentrations (data not shown).

Agonist-induced oscillations in $[Ca^{2+}]_i$ are a well documented feature in non-excitable cells. The characteristics of the oscillations vary greatly between different cell types, and in some cells these characteristics appear to be dependent on the agonist used to elicit the response (reviewed in Fewtrell, 1993). The oscillations observed in the present study in response to ACh were characterised by an extremely rapid rise in $[Ca^{2+}]_i$ followed by an equally rapid decline to basal levels (Figure 7.7). This regular spiking behaviour is very similar to that observed in vasopressin-stimulated hepatocytes (Rooney *et al*, 1989; Woods *et al*, 1986), histamine-stimulated endothelial cells (Jacob *et al*, 1988), and both pancreatic acinar and avian salt gland cells stimulated by cholinergic agonists (Jacob *et al*, 1988; Tsunoda *et al*, 1990). Further, ACh-stimulated $[Ca^{2+}]_i$ oscillations in bovine zfr cells closely resemble those seen in AII-stimulated bovine zg cells (Johnson *et al*, 1989),

but are markedly different from AII-stimulated responses in rat zg cells (Quinn *et al*, 1988). The Ca^{2+} transients in the latter preparation were highly asymmetric, with an initial sharp increase in $[Ca^{2+}]_i$ followed by a much slower decay during which the next oscillation occurred (Quinn *et al*, 1988).

The frequency of ACh-induced $[Ca^{2+}]_i$ oscillations appeared to be dose-related. No oscillatory behaviour was observed at low (10^{-6}M) concentrations of ACh. At 10^{-5}M ACh oscillations were clearly discernible, with an average period of around 30 sec, while at 10^{-4}M ACh, oscillations appeared to fuse together, with very rapid changes in $[Ca^{2+}]_i$ superimposed over an elevated baseline. Although insufficient data were available for a rigorous statistical analysis, a dose-dependent trend is clearly evident. This dose-dependent frequency modulation appears to be a general feature of those cell types exhibiting sharp Ca^{2+} spiking behaviour, but is rarely seen in cells in which Ca^{2+} oscillations are more sinusoidal in shape (Fewtrell, 1993).

In contrast to the $[Ca^{2+}]_i$ spiking behaviour induced by ACh, AII-stimulated $[Ca^{2+}]_i$ responses in bovine zfr cells were more homogeneous, and $[Ca^{2+}]_i$ oscillations were not observed at any AII concentration. This was surprising, since AII is known to stimulate Ca^{2+} oscillatory behaviour in individual rat (Quinn *et al*, 1988) and bovine (Johnson *et al*, 1989) zg cells. In common with ACh, however, a dose-dependent trend in the pattern of the $[Ca^{2+}]_i$ response to AII was observed, with higher doses of AII resulting in more sustained elevations of $[Ca^{2+}]_i$. It is possible that the doses of AII used in the present study were not low enough to induce $[Ca^{2+}]_i$ oscillations; it has been shown that in individual rat zg cells, $[Ca^{2+}]_i$ oscillations only occur at low doses of AII (picomolar), while higher (nanomolar) concentrations of AII produce Ca^{2+} responses similar to the profiles observed here (Quinn *et al*, 1988). This suggests that $[Ca^{2+}]_i$ oscillations in zg cells are regulated by the degree of receptor activation, and could explain the failure of AII to induce $[Ca^{2+}]_i$ oscillations in the present study.

Studies of AII effects on $[Ca^{2+}]_i$ in individual bovine zg cells have established that the initial increase in $[Ca^{2+}]_i$ in response to AII occurs through the mobilisation of intracellular Ca^{2+}, while the subsequent sustained Ca^{2+} increase (and $[Ca^{2+}]_i$ oscillations) is dependent on the presence of extracellular Ca^{2+} (Johnson *et al*, 1989). In populations of bovine zfr cells stimulated with AII, this 'late' Ca^{2+} response was much reduced in the absence of extracellular Ca^{2+} (Walker *et al*, 1991). It is possible therefore that the dose-dependency of the $[Ca^{2+}]_i$ response to AII observed in the present study reflects dose-dependent effects of AII on Ca^{2+} influx. This could occur through the PKC-mediated

phosphorylation of plasma membrane Ca^{2+} channels or potentially by direct effects of inositol phosphates on Ca^{2+} influx (Irvine & Moor, 1986).

The mechanism of agonist-induced $[Ca^{2+}]_i$ oscillations is unknown at present, and various different theories have been proposed. While such theories are generally consistent with the behaviour of one or two cell types, the wide variety of oscillation patterns observed in different cells has precluded the formation of a unifying model. The model proposed by Meyer and Stryer (1988) assumes that $[Ca^{2+}]_i$ oscillations result from the periodic release of intracellular Ca^{2+} due to fluctuations in the level of $Ins(1,4,5)P_3$. This is proposed to occur via a positive feedback by Ca^{2+}, released by $Ins(1,4,5)P_3$, on PLC. This positive feedback system results in a sharp increase in $[Ca^{2+}]_i$, which is terminated by sequestration of intracellular Ca^{2+} into an $Ins(1,4,5)P_3$-insensitive store. The next oscillation then occurs after replenishment of the $Ins(1,4,5)P_3$-sensitive store. Another popular model (Berridge & Galione, 1988) holds that fluctuations in $[Ca^{2+}]_i$ occur over a constant non-oscillating level of $Ins(1,4,5)P_3$. $Ins(1,4,5)P_3$ is thought to stimulate a constant release of Ca^{2+} from intracellular stores, which are constantly replenished by influx of extracellular Ca^{2+}. The Ca^{2+} released into the cytosol is sequestered into a Ca^{2+}-sensitive store, and thus $[Ca^{2+}]_i$ remains stable. However, when this Ca^{2+}-sensitive store is full, then $[Ca^{2+}]_i$ rises sharply producing a sharp peak in $[Ca^{2+}]_i$ which begins to decline when the Ca^{2+}-sensitive store is depleted. The next oscillation occurs on replenishment of this store.

In the present study, the frequency of ACh-induced $[Ca^{2+}]_i$ oscillations appeared to be dose-related. Both models discussed would allow for dose-dependent oscillation frequency modulation by the degree of receptor activation. Although both models propose intracellular Ca^{2+} as the source of Ca^{2+} for oscillations, the Ca^{2+}-induced Ca^{2+} release model requires that extracellular Ca^{2+} replenish the $Ins(1,4,5)P_3$-sensitive store (Berridge & Galione, 1988). If this were true, ACh-induced oscillations in bovine zfr cells would be expected to show a requirement for extracellular Ca^{2+}. In fact, ACh-induced $[Ca^{2+}]_i$ oscillations cease immediately on removal of extracellular Ca^{2+} (Williams, Gillespie, Skimming, Davies, Clyne, Kelly, Walker and Bird, unpublished observations). A similar effect has been reported in AII-stimulated zg cells (Johnson *et al*, 1989).

It is unknown at present why some agonists induce $[Ca^{2+}]_i$ oscillations and others do not. A model in which agonist receptors themselves regulate Ca^{2+} spiking activity has been proposed (Cobbold *et al*, 1991; Cuthbertson & Chay, 1991). This model proposes that PLC is only activated once a certain threshold of G protein activation has been reached,

resulting in a sudden burst of PLC activity, Ins(1,4,5)P_3 release, and consequent sharp increase in $[Ca^{2+}]_i$. Activation of PKC by Ca^{2+} and DAG is thought to lead to negative feedback on the earlier activation steps, leading to termination of the Ca^{2+} spike. Consistent with this hypothesis is the observation that inhibition of PKC in hepatocytes results in a longer half-time of decay of the Ca^{2+} spike (Woods *et al*, 1986). Phorbol ester treatment, which would therefore be expected to increase to half-time of spike decay, actually results in inhibition of $[Ca^{2+}]_i$ oscillations, and this has been interpreted as reflecting deactivation of agonist receptors and/or G proteins (Cobbold *et al*, 1991). This model therefore provides a mechanism by which a relatively weak agonist in bovine zfr cells, such as ACh, can initiate Ca^{2+} oscillations, whereas a stronger more efficacious agonist such as AII cannot.

In conclusion, the results presented in this chapter demonstrate different profiles of $[Ca^{2+}]_i$ behaviour in response to AII and ACh, with oscillations in $[Ca^{2+}]_i$ being described for the first time in adrenocortical inner zone cells. The relationship between oscillation frequency and steroid output, and the mechanisms by which oscillations are generated, remain to be established. Likewise, the physiological role of $[Ca^{2+}]_i$ oscillations in adrenocortical cells is unknown. Clearly, at low agonist concentrations, the generation of $[Ca^{2+}]_i$ oscillations would represent a significant improvement in signal:noise ratio compared with a small increase in $[Ca^{2+}]_i$, which may be difficult to distinguish from basal levels.

CHAPTER 8

GENERAL DISCUSSION

Contrary to the earlier view that cortisol and aldosterone secretion from the adrenal cortex is controlled exclusively by the peptide hormones ACTH and AII respectively, it is now clear that many different agonists are capable of modulating steroid secretion from experimental adrenocortical preparations. These agonists may represent classic autonomic neurotransmitters, such as acetylcholine or noradrenaline, peptidergic transmitters such as vasoactive intestinal peptide, endocrine hormones such as insulin or AII, or cytokines such as the interleukins or transforming growth factors. In general terms, significantly more information is available regarding agonist effects on zg cell function than on zfr cell function, and this probably reflects the importance of the renin-angiotensin-aldosterone system in the control of salt and water balance, and pathogenesis of hypertension and heart failure (reviewed in Hall *et al,* 1990; Schelling *et al,* 1991; Lindpainter & Ganten, 1991; Tan *et al,* 1991). However, given the crucial importance of glucocorticoids in the stress response and the maintenance of normal protein/carbohydrate metabolism and immune function, a fuller understanding of the mechanisms regulating cortisol secretion is clearly important. The potential effects of AII on inner zone cells are especially important in light of recent developments in the pharmacology of non-peptide AII antagonists, which are currently being developed as therapeutic agents for the treatment of hypertension and congestive heart failure (Timmermans *et al,* 1993). It will be important not to overlook possible effects of such antagonists on glucocorticoid secretion. The aim of this thesis was to compare and contrast the cellular mechanisms involved in the stimulation of zfr cell steroidogenesis by two different adrenocortical agonists, ACh and AII. As discussed in sections 1.5 and 1.6, both agonists have been shown to stimulate cortisol secretion in various species, and both have the potential to regulate steroidogenesis in the inner zones in vivo, although they may access the steroidogenic cells by different mechanisms. ACh is thought to be released from cholinergic nerve terminals within the cortex, while AII is formed via the renin-angiotensin system, either outwith the adrenal gland, or locally within the gland itself (section 1.3.4).

The model chosen for this study was a primary culture of bovine adrenocortical zfr cells. The reasons for this choice were three-fold. Firstly, the ease of obtaining fresh abattoir

material, coupled with the clear zonation of the bovine adrenal gland, allow the isolation of zfr cells at extremely high yield and purity. Secondly, this model has previously been well characterised with respect to ultrastructure and serum requirement, and shown to retain the steroidogenic responsiveness of freshly isolated cells to ACh and AII (Williams *et al,* 1989; Walker *et al,* 1990). Thirdly and most importantly, the available evidence suggests that cultured bovine zfr cells may be a more relevant model of the human inner adrenocortical zone compared with other commonly used preparations, at least with regard to AII: both bovine and human zfr cells respond to AII with an increase in cortisol secretion (Hadjian *et al,* 1984a; McKenna *et al,* 1978), and this effect can be mimicked by a combination of phorbol ester and calcium ionophore suggesting the involvement of the calcium messenger system in both species (Bird *et al,* 1992a; Laird *et al,* 1991). By contrast, rat and ovine zfr cells do not respond steroidogenically to AII, although both species show a clear stimulation of PtdIns hydrolysis in response to AII (Whitley *et al,* 1982; Viard *et al,* 1990). The effects of ACh in isolated human adrenal zfr cells have not been studied, although evidence exists to support a role of ACh in regulating human adrenocortical steroidogenesis in vivo (Stern *et al,* 1989).

In characterising this bovine zfr cell model, the steroidogenic responsiveness of cells to ACh and AII was examined on each day of primary culture (Chapter 3). This investigation was carried out in order to find the time of peak responsiveness of cells to ACh and AII, since previous studies have found that the ability of cultured adrenocortical cell preparations to respond steroidogenically to agonists varies according to culture time (Walker *et al,* 1988; Kawamura *et al,* 1984; Williams *et al,* 1989). Although the steroidogenic and second messenger responses to all agonists studied (AII, ACh, ACTH and adrenaline) were maximal following 48 - 72 h of primary culture, a clear difference in the ability of cells to respond to these agents was observed on day 2 of culture. While increased steroidogenic and second messenger responses to the adenylate cyclase agonists ACTH and adrenaline were observed at this time, the phospholipase C agonists AII and ACh were generally unable, or only weakly able, to stimulate cortisol secretion on culture day 2, even though enhanced second messenger (phosphoinositol) responses were observed at this time. Thus, bovine zfr cells appear to reversibly uncouple steroidogenesis from the activation of PLC in response to AII and ACh following 24 h in primary culture. Evidence was presented that this temporary loss of steroidogenic responsiveness to PLC agonists may be due to a defect in the protein kinase C-mediated transduction of the second messenger signals to the activation of steroidogenesis.

Although uncoupling of agonist receptor-PLC interactions is a well documented phenomenon, implicated in the mechanism of desensitisation, the uncoupling observed in

the present study was more unusual, in that the receptor-PLC interaction was preserved, but the second messengers formed were unable to stimulate steroidogenesis. The defect was selective for PLC agonists, since the steroidogenic and second messenger responses to ACTH and adrenaline were enhanced on day 2. There are several implications of this observation. Firstly, these results highlight the need for a careful optimisation of any new cell culture system. In this case, experiments performed on day 2 of primary culture would give erroneous results, and could lead to the conclusion that PLC agonists are not steroidogenic in this system. Secondly, the demonstration that steroidogenesis can become uncoupled from PLC activation may explain apparently anomalous results from other in vitro adrenocortical preparations. Ovine zfr cells in primary culture responded to AII with an increase in PLC activity, $^{45}Ca^{2+}$ uptake and $[Ca^{2+}]_i$, but do not respond steroidogenically to the peptide (Viard *et al,* 1990). As was the case for bovine zfr cells, treatment with PMA and A23187 failed to stimulate steroidogenesis in ovine zfr cells. Since these experiments were performed using day 4 primary cultured cells only, the possibility is raised that AII could stimulate steroidogenesis in ovine zfr cells following different periods of primary culture. Similar uncoupling of steroidogenesis from PLC activation has been demonstrated in 3-day primary cultured rat zfr cells stimulated with the PLC agonist vasopressin (Gallo-Payet *et al,* 1986). Again, investigation of agonist effects on different experimental days may produce different results.

The data presented in chapter 3 are clearly insufficient to determine whether reversible uncoupling in bovine zfr cells occurs in vivo, or is simply a cell culture artifact. Neither do these results provide any indication of the physiological significance, if any, of this dissociation. To speculate, the ability of bovine zfr cells to uncouple PLC activation from stimulation of steroidogenesis may represent a mechanism whereby the stimulatory effects of PLC agonists can be diverted from the steroidogenic pathway to stimulation of some other cell function, for example, cell growth. AII is known to stimulate [^{3}H]thymidine incorporation into unpurified cultured bovine adrenocortical cells (Gill & Simonian, 1977; Simonian & Gill, 1979a) and purified bovine zfr cells in primary culture (Clyne *et al,* 1993), although no information is available regarding the possible mitogenic effects of ACh in these cells.

A major component of this thesis comprised the classification of the bovine zfr cell receptors for ACh and AII (Chapters 4 and 5). Estimation of the affinity of a number of selective antagonists of ACh and AII by Schild analysis provided evidence to suggest that ACh and AII stimulate cortisol secretion from bovine zfr cells via activation of M_3 muscarinic and AT_1 receptors respectively. However, while the pA_2 values for muscarinic antagonists were typical of previously characterised M_3 receptors, evidence

was presented to suggest that the bovine zfr AII receptor differs from previously characterised AII receptors. The selective inhibition of AII-stimulated cortisol secretion and [^{3}H]phosphoinositol production by losartan clearly indicates involvement of the AT_1 receptor (Bumpus *et al*, 1991). However, since losartan was significantly less potent in zfr cells than at AT_1 receptors in other systems, it was suggested that the bovine zfr receptor may represent a distinct subtype of AT_1 receptor, or a mixture of different AT_1 receptor subtypes with different affinities for losartan. The latter possibility was supported by the demonstration of two distinct losartan-sensitive ^{125}I-AII binding proteins in zfr cell membranes fractionated by isoelectric focusing. It would clearly be of interest therefore to compare the ability of losartan to displace ^{125}I-AII binding to each AII binding protein.

Although AT_1 receptor subtypes exist in rat and mouse tissues (Iwai & Inagami, 1992; Sasamura *et al*, 1992), similar investigations of AT_1 receptor heterogeneity have not yet been performed in bovine species. The rat AT_1 receptor subtypes appear to show a ten-fold difference in their ability to bind angiotensin I (Kakar *et al*, 1992), although it is not clear at present if AT_{1A} and AT_{1B} receptors differ in their affinity for losartan (Timmermans *et al*, 1993). However, in relation to the situation in bovine species, binding studies from several laboratories have demonstrated a reduced potency of losartan in inhibiting ^{125}I-AII binding to bovine adrenal cortex preparations (Balla *et al*, 1991; Ouali *et al*, 1992; Sasaki *et al*, 1991). The results presented in chapter 5 confirm these observations, and show that this reduced binding affinity of losartan in the bovine adrenal cortex is reflected by a reduced potency in functional assays.

If, as has been suggested above, bovine zfr cells contain two distinct AT_1 receptors with unequal affinity for losartan, then the possibility arises that these subtypes may be coupled to different cellular responses, which may therefore be expected to be differentially inhibited by losartan. In fact, losartan was around two orders of magnitude more potent in inhibiting the mitogenic response to AII in cultured bovine zfr cells (ID_{50} = 5 x 10^{-8}M) than the steroidogenic response (ID_{50} = 3.1 x 10^{-6}M) (Clyne *et al*, 1993). Thus, the bovine zfr cell preparation utilised here may prove a useful model for future investigations of AT_1 receptor heterogeneity.

Having identified the receptor subtypes through which ACh and ACH stimulate cortisol secretion, the effects of these agents on PtdIns hydrolysis and $[Ca^{2+}]_i$ mobilisation were assessed. Previous studies have suggested that ACh and AII stimulate a common PLC enzyme (Bird *et al*. 1990a), leading to mobilisation of calcium from a common intracellular pool (Walker *et al*, 1991). The data presented in Chapter 6 extend these

observations, establishing that a common pool of PtdIns is metabolised in response to both ACh and AII. Although this is consistent with a common mechanism of action for ACh and AII in zfr cells, attempts to confirm this hypothesis through investigation of the possible additive effects of ACh and AII were unsuccessful: their effects in combination were only partially additive with respect to both cortisol secretion and PLC activity. This could reflect a heterologous desensitisation effect, as previously reported to result in partial additivity of bradykinin and vasopressin responses in WRK-1 cells (Monaco *et al*, 1990). However, the demonstration that ACh and AII stimulate the breakdown of a common PtdIns pool, coupled with the very close correlation between ACh- and AII-stimulated cortisol secretion and PLC activity (Figure 6.3), lend support to the hypothesis of a common mechanism of action in bovine zfr cells.

The final experimental section of this thesis compared the abilities of ACh and AII to elevate $[Ca^{2+}]_i$ in zfr cells. The main conclusions to emerge from this work were as follows:

(i) ACh is capable of inducing oscillations in $[Ca^{2+}]_i$ in bovine zfr cells.

(ii) The frequency of ACh-induced $[Ca^{2+}]_i$ oscillations appeared to be concentration-dependent.

(iii) AII does not stimulate $[Ca^{2+}]_i$ oscillations in bovine zfr cells.

As discussed in Chapter 7, several contradictory models have been proposed to explain the mechanisms of agonist-induced $[Ca^{2+}]_i$ oscillations. The data presented in the present study appears to be most consistent with the receptor-controlled oscillator model (Cobbold *et al*, 1991), which predicts that sustained PLC activity inhibits the generation of Ca^{2+} spikes. This model could therefore explain the failure of AII to induce $[Ca^{2+}]_i$ oscillations, since the concentrations of AII used here were somewhat higher than those reported to stimulate oscillatory activity in adrenocortical zg cells (Quinn *et al*, 1988; Johnson *et al*, 1989). Further study will be required to resolve this question.

The bovine zfr cell model used in this study is clearly a useful preparation for the investigation of adrenocortical agonist receptors and effectors. Data presented in this thesis has extended knowledge of ACh and AII effects on the bovine adrenal cortex at the cellular level. An important question, however, concerns the physiological relevance of these agonists in the adrenal. Under which conditions may ACh and AII gain access to the cortex, and what are the likely effects of their release within the gland? Perhaps more

importantly, how do intra-adrenal ACh and AII interact, both with each other, and with other stimulatory factors? Under conditions of stress, ACTH is released from the hypothalamus, and the sympathetic nervous system is activated. Sympathetic splanchnic nerve stimulation results in release of ACh within the medulla, although it is not known as yet if release of ACh within the cortex occurs. This seems likely, however, given the presence of cholinergic nerve terminals, derived from the splanchnic nerve, within the cortex (Charlton *et al,* 1991), and the observation that adrenal responses to splanchnic nerve stimulation in cattle are very similar to those in response to infusion of ACh (Edwards & Jones, 1987; Jones *et al,* 1991). Although AII can reach the adrenal cortex through the action of renin secreted from the kidney, the evidence for an intra-adrenal renin-angiotensin system reviewed in section 1.3.4. suggests that AII may also be formed locally within the gland. Since renin activity in cultured bovine zg cells is potently stimulated by catecholamines (Gupta *et al,* 1992), it is possible that catecholamines, released within the cortex on splanchnic nerve stimulation (Charlton *et al,* 1992; Gilchrist *et al,* 1993), may stimulate adrenocortical AII formation. The juxtaposition of medullary and cortical cells in several species (section 1.1.4) raises the possibility of an indirect stimulation of adrenocortical AII formation by ACh: medullary catecholamines formed on splanchnic nerve stimulation may reach the cortex and stimulate renin. Thus, evidence exists to support the formation of both ACh and AII within the adrenal cortex under conditions of increased splanchnic nerve activity.

The effects of ACh infusion on adrenal gland function in cattle *in vivo* have been studied by Jones *et al* (1991). These included an decrease in adrenal vascular resistance, indicating vasodilatory effects of ACh which could enhance the delivery of other adrenocortical agonists, such as AII, to the steroidogenic cells. Medullary responses included release of catecholamines, CRF and enkephalins, all of which could potentially modify steroid output if they were able to reach the cortical cells. The authors also suggested that since adrenal responses to ACh infusion differed subtly from those in response to splanchnic nerve stimulation, other (as yet unidentified) neurotransmitters are likely to be released from splanchnic nerve terminals. Thus, while it is clear that both ACh and AII are capable of stimulating steroid secretion in many species, it seems probable that the physiological relevance of these agents in the adrenal cortex may depend on complex interactions with other agonists.

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APPENDIX 1: PUBLICATIONS

The following publications arose in full or part as a result of the work described in this thesis:

Papers

Walker, S.W., Lightly, E.R.T., **Clyne, C.D.**, Williams, B.C. & Bird, I.M. (1991). Adrenergic and cholinergic regulation of cortisol secretion from the zona fasciculata/reticularis of bovine adrenal cortex. *Endocrine Res.* **17**:235-256.

Bird, I.M., **Clyne, C.D.**, Williams, B.C. & Walker, S.W. (1992). Further characterisation of the steroidogenic responsiveness of purified zona fasciculata/reticularis cells from bovine adrenal cortex before and after primary culture: changing responsiveness to phosphoinositidase C agonists. *J. Endocrinol.* **133**:21-28.

Clyne, C.D., Williams, B.C., Walker, S.W. & Bird, I.M. (1992). Studies of hormone-sensitive and -insensitive pools of phosphoinositides in cultured bovine zona fasciculata/reticularis cells: evidence that acetylcholine and angiotensin II stimulate the breakdown of a common pool of phosphoinositides. *Biochem. Pharmacol.* **44**:441-446.

Clyne, C.D., Nicol, M.R., MacDonald, S., Williams, B.C. & Walker, S.W. (1993). Angiotensin II stimulates growth and steroidogenesis in zone fasciculata/reticularis cells from bovine adrenal cortex via the AT_1 receptor *Endocrinology* **132**:2206-2212.

Clyne, C.D., Walker, S.W. & Williams, B.C. (1994). Characterisation fo the muscarinic receptor subtype mediating acetylcholine-induced cortical secretion from bovine adrenocortical fasciculata/reticularis cells. *Biochem. Pharmacol.* (In Press).

Abstracts

Strachan, M.J.W., **Clyne, C.D.**, Walker, S.W., Williams, B.C. & Bird, I.M. (1990). A comparison of the effects of angiotensin II, acetylcholine and vasopressin on cortical secretion, phosphoinositidase C activity and cytosolic [Ca^{2+}] in cultured bovine adrenocortical fasciculata/reticularis (zfr) cells. *J. Endocrinol.* **124**(suppl), Abstract 61.

Clyne, C.D., Bird, I.M., Kelly. J.S., Walker, S.W. & Williams, B.C. (1991). Acetylcholine and angiotensin II stimulate breakdown of a common pool of phosphoinositides in cultured bovine fasciculata/reticularis cells. *Br. J. Pharmacol.* **102**(suppl), 207P.

Bird, I.M., **Clyne, C.D.**, Lightly, E.R.T., Walker, S.W & Williams, B.C. (1991). Stimulatory effects of angiotensin II and acetylcholine on bovine adrenocortical cells before and after primary culture: evidence for reversible uncoupling of phosphoinositidase C activation and steroidogenesis. *J. Endocrinol.* **129**(suppl), Abstract 54.

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Nicol, M., **Clyne, C.D.**, MacDonald, S., Davies, E., Williams, B.C. & Walker, S.W. (1992). Angiotensin II stimulates cortisol secretion and cell growth via the AII_1 receptor subtype in cultured inner zone cells from bovine adrenal cortex. *J. Endocrinol.* **132** (suppl), Abstract 102.

Clyne, C.D., Walker, S.W., Nicol, M.R., Kelly, J.S. & Williams, B.C. (1993). Characterisation of the muscarinic cholinoceptor subtype mediating ACh-induced cortisol secretion from bovine adrenocortical inner zone cells in primary culture. *Br. J. Pharmacol.* **108**(suppl), 234P.

Clyne, C.D., Williams, B.C., Nicol, M.R., Kelly, J.S. & Walker, S.W. (1993). Characterisation of the receptor mediating the steroidogenic and mitogenic effects of angiotensin II in bovine adrenocortical inner zone cells in primary culture. *Br. J. Pharmacol.* **108**(suppl), 188P.

ENDOCRINE RESEARCH, 17(1&2), 237-265 (1991)

ADRENERGIC AND CHOLINERGIC REGULATION OF CORTISOL SECRETION FROM THE ZONA FASCICULATA/RETICULARIS OF BOVINE ADRENAL CORTEX

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ABSTRACT

Inner zone cells, isolated from bovine adrenal cortex, secrete cortisol in response to both adrenergic and cholinergic agonists.

The response to adrenaline (and other catecholamines) appears during culture, is evident by 24 h and reaches a maximum by 48-72 h, but is absent in freshly isolated cells. Pre-incubation of cultured cells with adrenaline leads to homologous desensitisation; the possibility that this may explain the absent response in freshly isolated cells is discussed. Cells show a dose-dependent cyclic AMP response but no increased membrane phosphoinositide turnover. In agreement, cortisol secretion is blocked by β-receptor, but not α-receptor, antagonists. Schild analysis established that the response occurs through binding to a β_1-receptor subtype, consistent with adrenergic innervation as opposed to an effect of circulating catecholamines.

In contrast, cortisol secretion to AcCh was present in both freshly isolated cells and those in culture, reaching a maximum by 48-72 h in culture. The response was specifically blocked by muscarinic, but not nicotinic, antagonists. No effect on cyclic AMP formation was observed, but dose-dependent stimulation of phosphoinositide turnover occurred. HPLC analysis of the time-course of appearance of ^{3}H-inositol labelled head

groups (from cells pre-labelled with ^{3}H-inositol) confirmed that AcCh activates a phosphoinositidase C. Intracellular Ca^{2+} oscillations were also measured from fura-2 loaded single cells in response to AcCh. Together with other pharmacological studies, these observations establish that AcCh acts through a M3 muscarinic receptor subtype in these cells.

The possible significance of these findings in vivo is discussed.

Introduction

Although adrenocorticotropic hormone is the principal regulator of glucocorticoid formation from the inner zone of the adrenal cortex, both adrenergic and cholinergic effects have been described.

Adenylate cyclase activity stimulated by catecholamines has been reported in membrane preparations from rat 494 corticosterone-secreting tumours (1,2) and in human adrenocortical carcinomas, though not in normal human adrenal cortex (3). Stimulation of aldosterone secretion has been reported from capsular explants of rat adrenal cortex in a perifusion system (4) and from 3-day cultures of bovine adrenocortical cells (5). Cortisol production has also been reported from a mixed bovine adrenocortical cell preparation in primary culture (6). Only recently has the stimulatory effect of catecholamines on glucocorticoid secretion, specifically from the inner zone, been studied (7). Where the second messenger response has been investigated, steroid

secretion appears to be mediated by adenosine 3',5' cyclic monophosphate (cyclic AMP) (6,7).

Cholinergic stimulation of adrenocortical steroidogenesis has also been found. Acetylcholine, but nct nicotine, stimulated steroid production from the perfused calf adrenal (8). Cortisol secretion has also been described from cultured bovine zona fasciculata/reticularis (zfr) cells in response to muscarinic, cholinergic agonists (9, 10). Evidence that membrane phosphoinositide turnover mediates the cholinergic response in bovine fasciculata/reticularis cells exists (11). More recently, activation of phosphoinositidase C by acetylcholine has been shown for bovine zfr cells (12).

In this article, we describe our recent work on the catecholaminergic and cholinergic regulation of cortisol secretion from inner zone cells of bovine adrenal cortex. The significance of these results to the situation in vivo is discussed.

Materials and Methods

The source of materials and details of cell isolation and primary culture of bovine zfr cells are described in Williams et al (13). The experimental details and material sources for the study of cortisol and cyclic AMP formation in response to catecholamines are reported in

Walker et al (7) and for subclassification of the β-receptor in Lightly et al (14). For the cholinergic response, the appropriate experimental methods and the source of materials is described in Walker et al (12).

Studies of changes in single cell [Ca^{2+}] were carried out using cells cultured on 2.5 cm diameter glass coverslips at a density of 150,000 cells/ml. Cells were loaded with Fura 2-AM (5 uM) for 2 h, and the cover slip then transferred to the perfusion chamber on the microscope stage. The chamber was perfused with pre-warmed (37°C) Krebs Ringer/Hepes buffer (pH7.4 with 2.5 mM $CaCl_2$), with or without acetylcholine as required. Cells were alternately excited at 350 and 380 nm, and the emitted light measured at 520 nm using a Nikon Diaphot inverted microscope.

Results

Cortisol responsiveness over the first 96 h in culture

Fig.1 illustrates the cortisol secretion of the bovine zfr cells measured over 1 h on day 1 (freshly collagenase-dispersed) and at 24 h intervals thereafter for 96 h in culture. The basal cortisol output is shown, together with the corresponding responses to maximally stimulatory doses of ACTH (10^{-9}M) and adrenaline (10^{-6}M). Also shown for comparison is the day-by-day response of the zfr cells to angiotensin II (AII) at a concentration

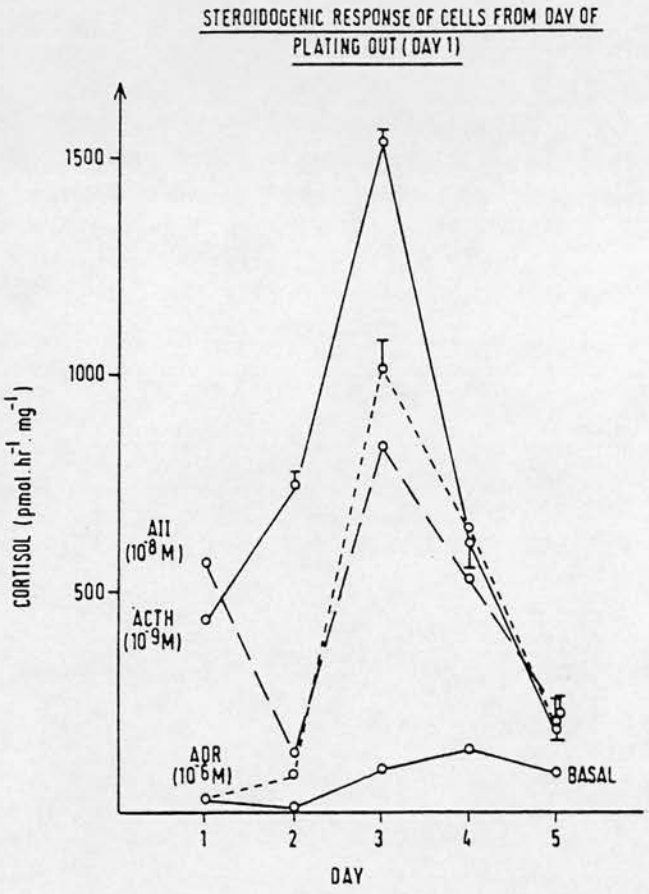


Fig 1: Day-by-day responses of bovine zfr cells to ACTH (—), AII (--) and adrenaline (-·-).

The cortisol output was measured over 1 h for freshly isolated cells (day 1) and at 24 h intervals thereafter for cells in monolayer culture. The basal (no agonist) response is shown, together with the responses to maximally stimulatory doses of ACTH (—), AII (--) and adrenaline (-·-). Each point is the mean±S.D. for 3 wells in a representative experiment.

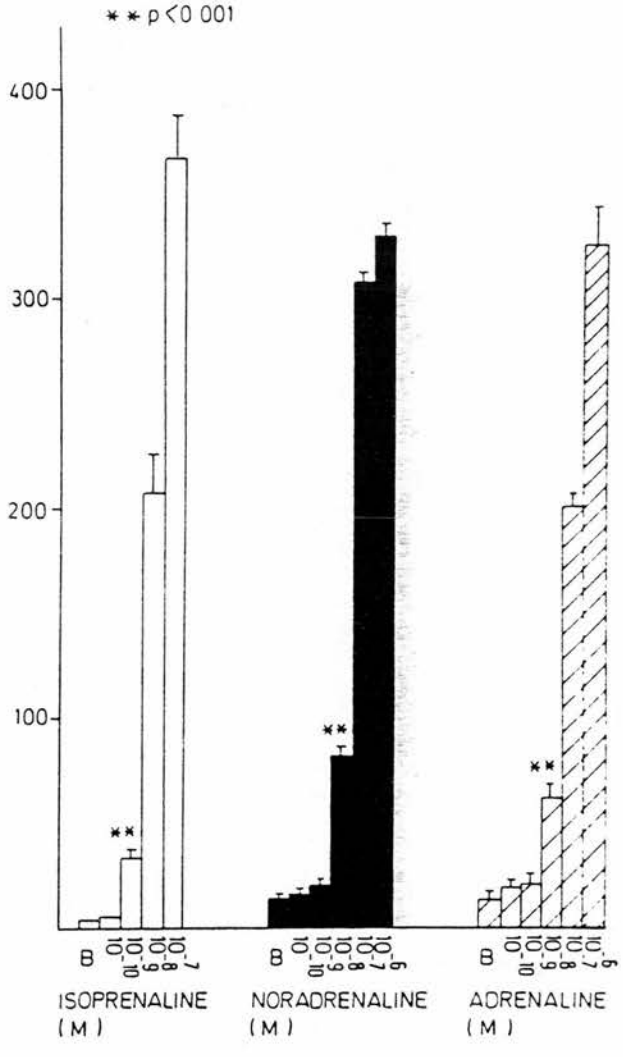


Fig 2: The cortisol and cyclic AMP response of cultured bovine zfr cells to catecholamines.

In Fig. 2A, the cortisol secretory response over 1 h (expressed as $pmol.h^{-1}.mg\ protein^{-1}$) is measured at 48 h in culture (day 3) for the three catecholamines, isoprenaline, noradrenaline and adrenaline. The basal response is shown for comparison. In Fig. 2B, the corresponding cyclic AMP production by the cells is shown. Each point is the mean±S.D. for 3 wells in a representative experiment.

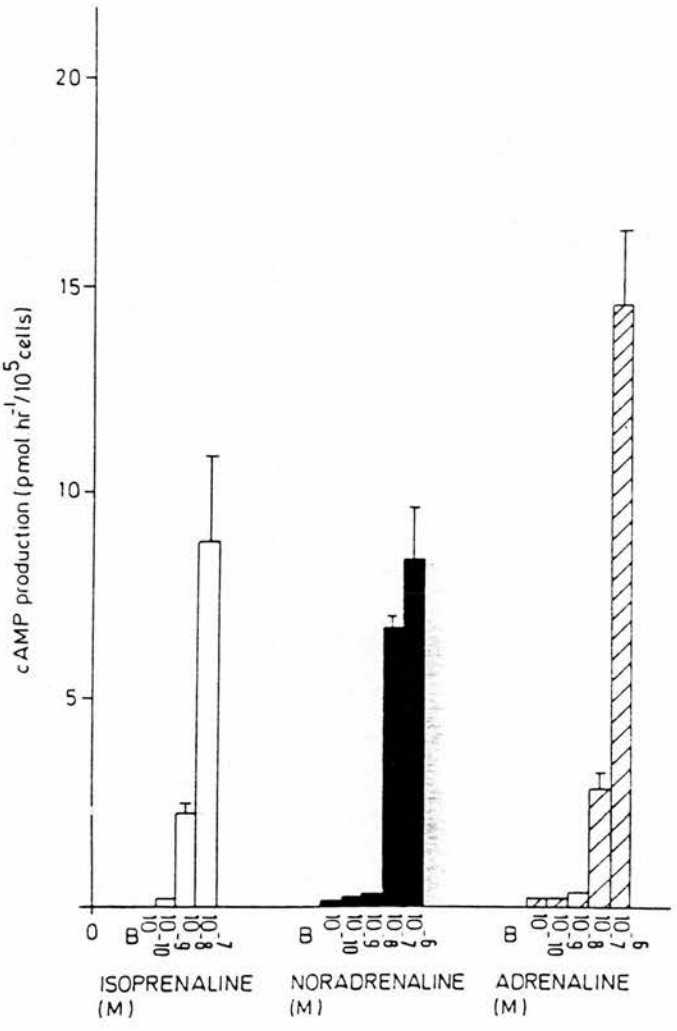


Fig. 2B

of 10^{-8}M; bovine zfr cells show a marked response to this agonist, which stimulates membrane phosphoinositide turnover (14).

Although freshly dispersed cells secrete cortisol to both ACTH and AII, there is no significant increase above basal secretion to adrenaline on day 1. After 24 h in

culture, however, the cells now respond to adrenaline. The cortisol secretory response to all three agonists peaks by 48 h and declines thereafter.

Cortisol and cyclic AMP response to catecholamines

Fig. 2A shows the cortisol response to increasing doses of isoprenaline, noradrenaline and adrenaline for cells after 48 h in primary culture. The mean ED_{50} for adrenaline was found to be 5.0×10^{-8}M (range $2.0 - 12.6 \times 10^{-8}$M; n=3). In Fig. 2B, the dose-dependent increase in medium cyclic AMP content in response to increasing doses of the same three agonists is also evident.

The failure of adrenaline to elicit any significant increase in ^{3}H-inositol-labelled total aqueous head group production from cells whose membrane phosphoinositides had previously been labelled to isotopic steady state with ^{3}H-inositol is shown in Fig. 3. The same figure uses AII as a positive control for the head group assay and also illustrates the corresponding lack of effect of AII on cyclic AMP formation. The ability of the β-receptor antagonist, propranolol, to completely block the effect of adrenaline on cortisol and cyclic AMP production and its lack of effect on the steroid and second messenger responses to AII is also shown.

Homologous desensitisation by adrenaline

Fig. 4 illustrates the effect of pre-incubating the cells with adrenaline (10^{-7}M) for different lengths of

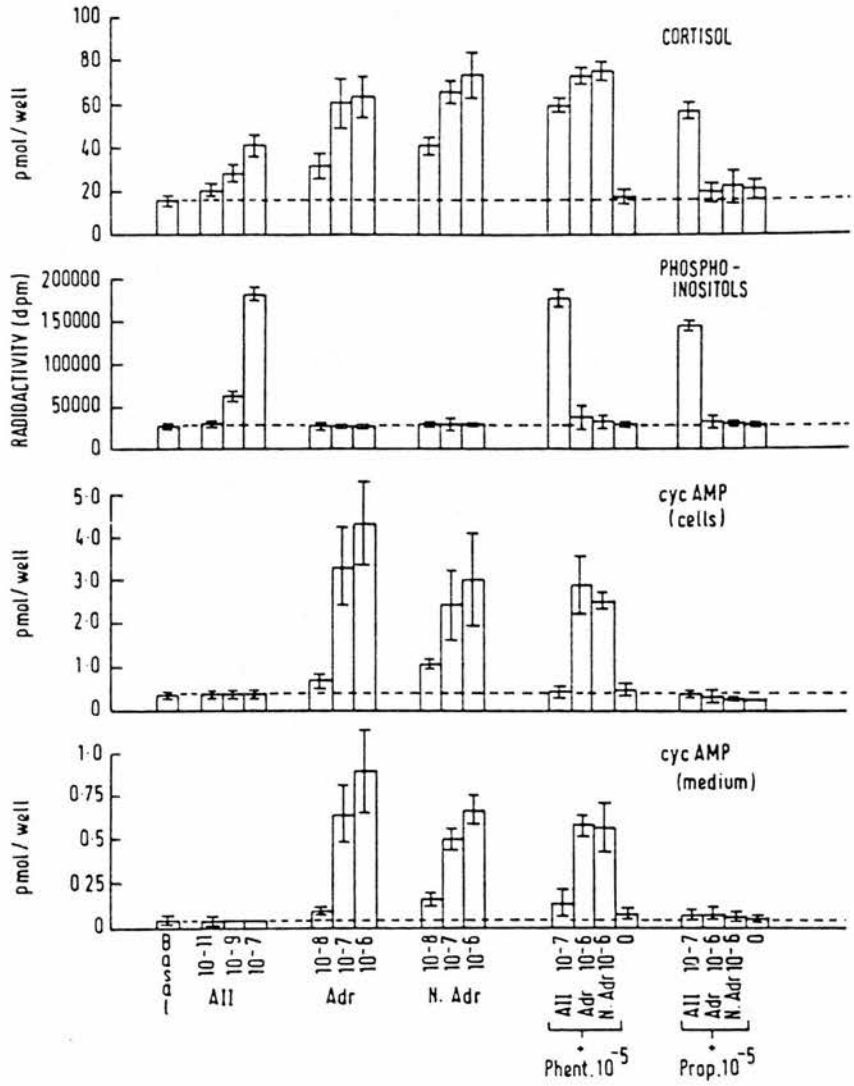


Fig 3: Effects of adrenaline, noradrenaline and AII on cortisol secretion, phosphoinositidase C activity and cyclic AMP accumulation.

Cells were incubated for 15 min with agonists and, where relevant, antagonists at the concentrations shown. For measurement of cortisol and cyclic AMP responses, incubation medium was removed at the end of this time and cortisol and cyclic AMP (medium) content determined by radioimmunoassay. Cellular cyclic AMP was also determined after washing and ethanol extraction. For monitoring phosphoinositidase C activity, [^{3}H]inositol-prelabelled cells were used and stimulation was carried out in the presence of Li^+ (10 mM). The total phosphoinositol fraction was then recovered and its radioactivity determined. Abbreviations: adrenaline (Adr), noradrenaline (N.Adr), phentolamine (Phent), propranolol (Prop). Results shown are the mean±S.D. of triplicate values from a single experiment

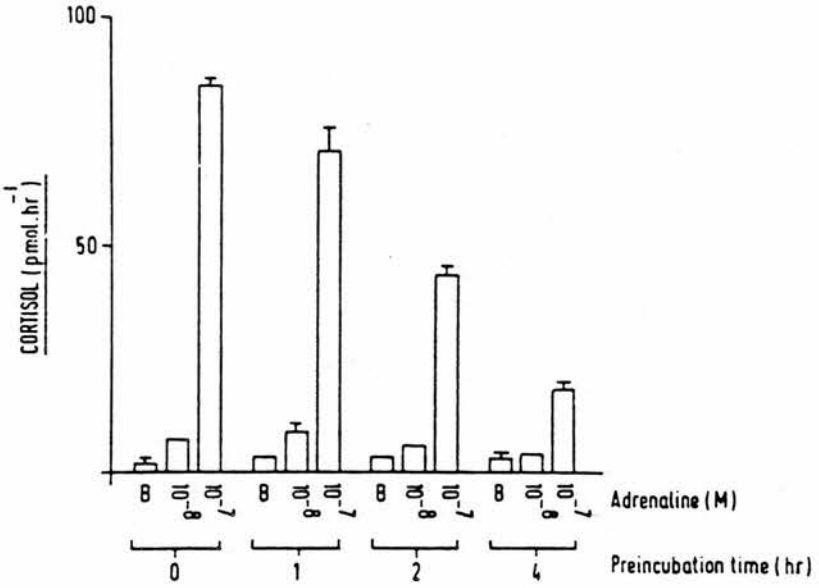


Fig 4: Homologous desensitisation of the adrenergic response.

Cells were pre-incubated with adrenaline (10^{-7}M) after 48 h in culture. At different times thereafter, the adrenaline was washed out and the cells challenged with zero (i.e basal), 10^{-8}M or 10^{-7}M adrenaline and the cortisol secretion over a 1 h period measured. Each bar is the mean±S.D. for 3 wells in a representative experiment (the error bar only being shown when sufficiently large to appear on the scale used). The '0' time response relates to cells which have not been previously exposed to adrenaline. Protein content of the wells was not affected by the pre-incubation.

time on a subsequent challenge to zero (basal), 10^{-8}M or 10^{-7}M adrenaline. A time-dependent homologous desensitisation of the cortisol response is evident.

Characterisation of the adrenergic receptor

Experiments with β- and α-receptor antagonists were consistent with adrenergic stimulation occurring through

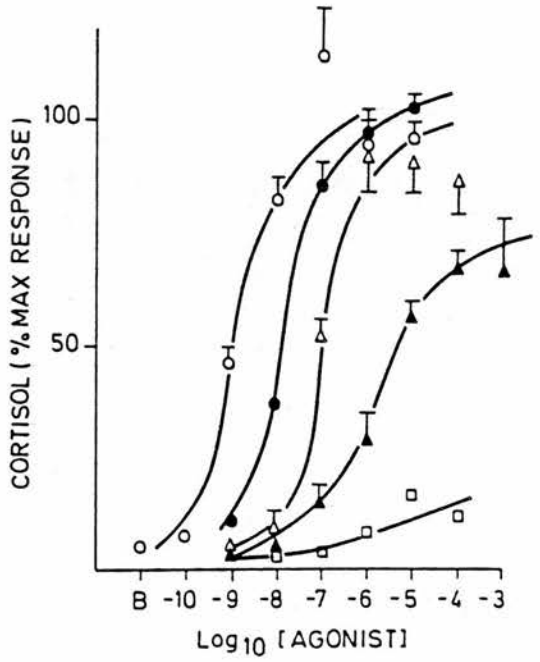


Fig 5: Dose response curves for cortisol secretion to different catecholaminergic agonists.

The cortisol response over 1 h was measured for cells at 48 h (day 3) in response to isoprenaline (○), noradrenaline (●), dobutamine (△), salbutamol (▲) and BRL37344A (□). B=basal cortisol production. Each point is the mean±S.D. for 3 wells.

a β-receptor in the bovine zfr cells (Fig.3., see also (7)). Dose-response curves for various selective β-agonists are shown for the zfr cells at 48 h in primary culture in Fig. 5. Isoprenaline, noradrenaline and dobutamine all acted as full agonists, whilst salbutamol, a full agonist at β_2 receptors, but a partial agonist at β_1 receptors (16), produced only 70% of the maximal response. The compound BRL37344 (Beecham Pharmaceuticals,

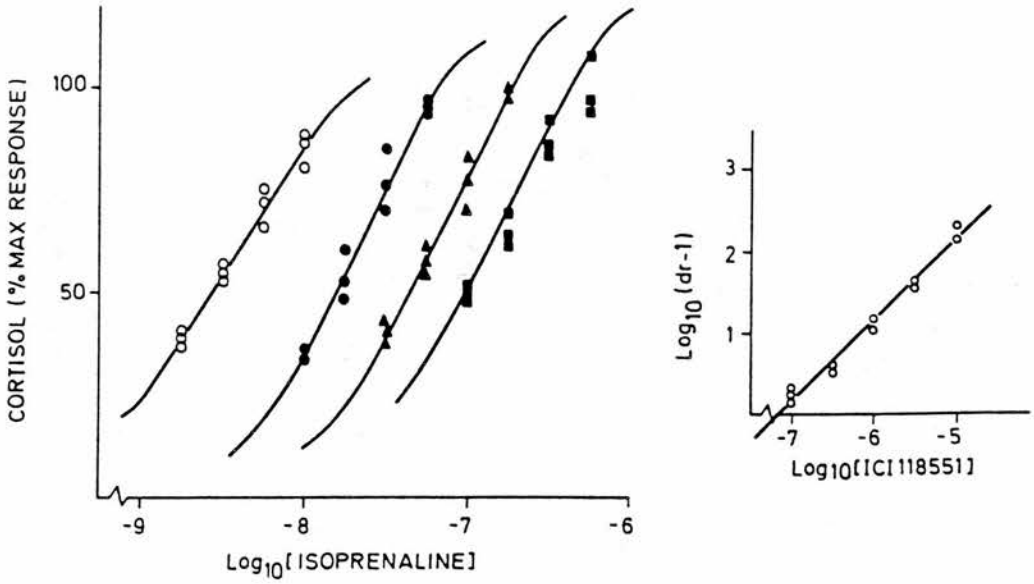


Fig 6: Schild analysis (ICI 118551)

Fig. 6A is a representative experiment showing cortisol secretion at 48 h (day 3) following stimulation with isoprenaline alone (o) and in the presence of increasing concentrations of ICI 118551 $10^{-6.5}$M (●), 10^{-6}M (▲) and $10^{-5.5}$M (■). Each point is the mean±S.D. for 3 wells. In Fig. 6B is shown the Schild regression - least squares fit of $\log_{10}$ (DR-1) versus $\log_{10}$ [ICI 118551], where DR = dose ratio. Cumulative data from 3 separate cell preparations.

Epsom, Surrey, U.K.), known to be a full agonist at β_3-receptors (17), was a poor agonist for the zfr cells. These results were consistent with the cortisol secretory effect of adrenaline occurring through the latter's binding to a β_1- receptor.

To confirm this preliminary characterisation, Schild plots were obtained using the selective β_1-antagonist, practolol and the selective β_2-antagonist, ICI 118,551

Table 1. Comparison of experimental and published data for practolol and ICI 118,551.

Antagonist	Experimental pA2	95% CL	Slope	95% CL	Published pA2
Practolol	6.85	6.67 7.06	0.9	0.84 0.96	6.80[18] (β1)
ICI 118,551	7.14	7.03 7.28	0.99	1.01 0.97	7.17[19] (β1) 9.26[19] (β2)

95% CL = 95% confidence limits. Original references are (18) and (19).

(ICI plc, Macclesfield, Cheshire, U.K.). Fig. 6 illustrates dose-response curves to isoprenaline in the presence of increasing concentrations of the β_2 antagonist, ICI 118,551; the inset figure shows the corresponding Schild plot derived from the linear portions of these dose-response curves. An identical approach was used to derive the Schild plot for the β_1-antagonist, practolol (not illustrated).

The calculated pA_2 values for practolol and ICI 118,551 from these Schild lots and the pA_2 values reported in the literature are summarised in table 1.

The effects of cholinergic agonists on cortisol secretion

In contrast to the absent adrenergic response, freshly collagenase-dispersed bovine zfr cells were

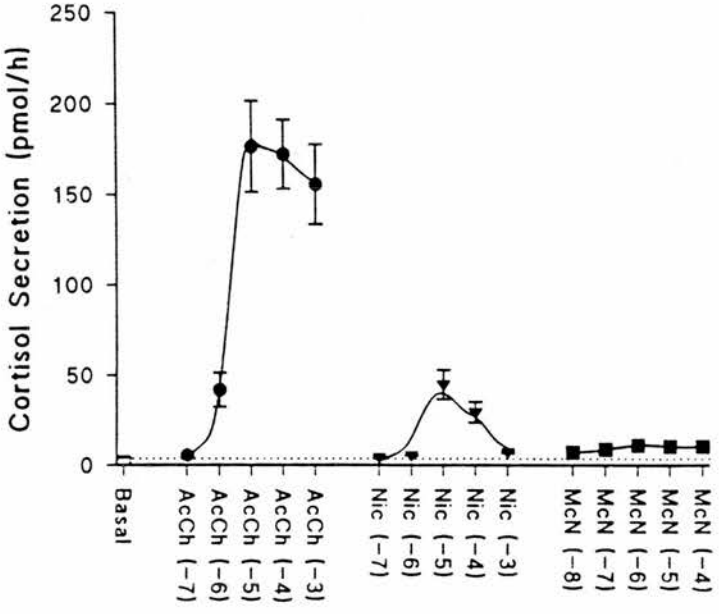


Fig 7: Cortisol response to AcCh, nicotine and McN-A-343.

Cortisol production (1 h incubation) for increasing doses of the 3 different agonists is shown (●= AcCh; ▼ = nicotine; ■ = McNeil-A-343). The basal cortisol production is shown as the single bar (labelled 'Basal'). Each point is the mean±S.D. for triplicate wells in a single experiment.

significantly stimulated (as compared to basal) by acetylcholine (12). As with ACTH and adrenaline (at later times in culture), cholinergic stimulation peaked by 48 h, declining thereafter. The ED_{50} with respect to acetylcholine was 1.2×10^{-6}M (range 3.7×10^{-7} to 3.7×10^{-6} M; n=4 experiments).

Fig. 7 displays dose-response curves to AcCh, nicotine and the M1 receptor agonist McN-A-343, from which it is evident that the response is muscarinic,

although not involving the M1 receptor. The small nicotinic response was inconsistent (slight stimulation in 2 out of 5 experiments, only), and cells showed morphological evidence of damage at the high doses necessary to elicit a response.

The effect of cholinergic antagonists on AcCh-stimulated cortisol secretion

Fig.8A shows a potent dose-dependent inhibition of the response to AcCh by the muscarinic receptor antagonist, atropine. Tubocurarine, a nicotinic antagonist, had little effect, except for incomplete inhibition at the highest doses. Also shown is the failure of propranolol to inhibit the AcCh response, thereby excluding the possibility that AcCh was releasing adrenaline from contaminating medullary cells and leading indirectly to cortisol secretion.

In Fig. 8B, dose-dependent inhibition of the response by hexahydro-sila-difenidol is shown. This antagonist has high anti-muscarinic potency at M3 receptors but low potency at M2 receptors, with intermediate potency at M1 receptors.

The second messenger response to AcCh

Previous studies have shown that there is no increase in cyclic AMP in zfr cells in response to AcCh. In contrast, there is evidence for increased ^{32}P-

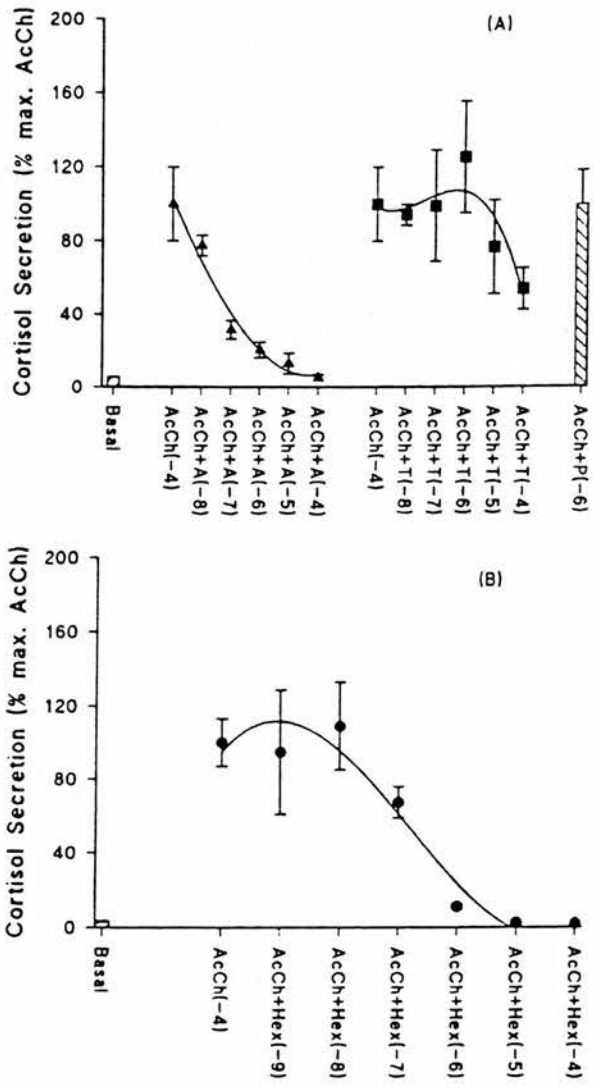


Fig 8: The effects of different antagonists on AcCh-stimulated cortisol secretion.

The cortisol response (1 h incubation) was measured for each agonist, antagonist or combination, expressing the secretion as a percentage of the response achieved by 10^{-4} M AcCh (100%).

Panel A shows the effect of the muscarinic antagonist, atropine (A) (▲) and the nicotinic antagonist, tubocurarine (T) (■). The basal response alone is shown as the single bar ('Basal'). The effect of propranolol (P) (at 10^{-6} M) on the AcCh-stimulated response is shown in the last bar of the figure.

Panel B shows the effect of the M3 antagonist, hexahydro-sila-difenidol (Hex) (●). The basal response alone is shown as the single bar ('Basal').

All values are the mean±S.D. for triplicate wells in a single experiment. In all cases, no effect of the antagonist alone on basal cortisol secretion was found.

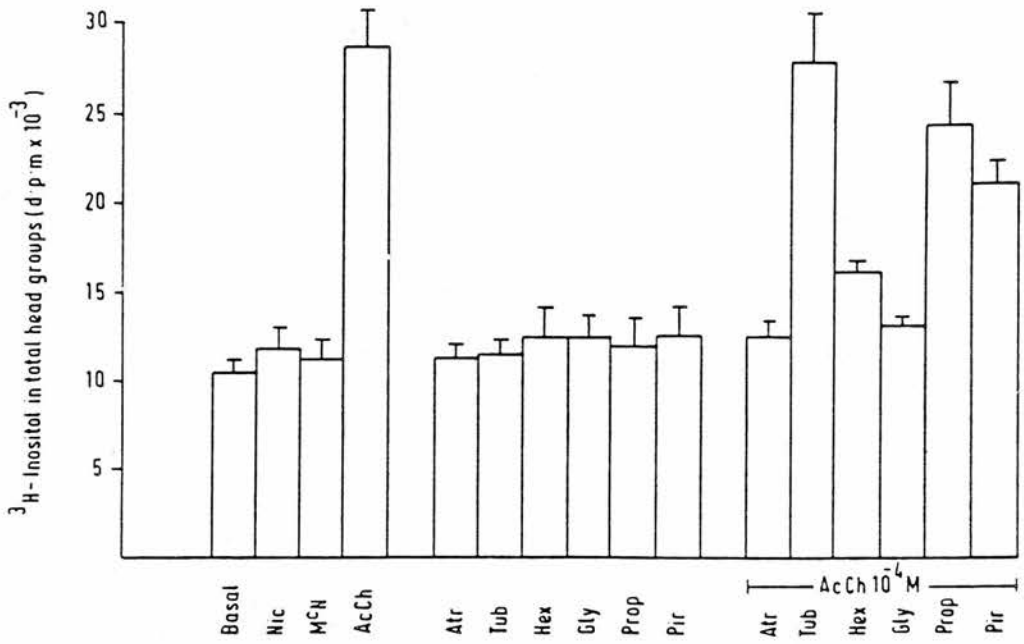
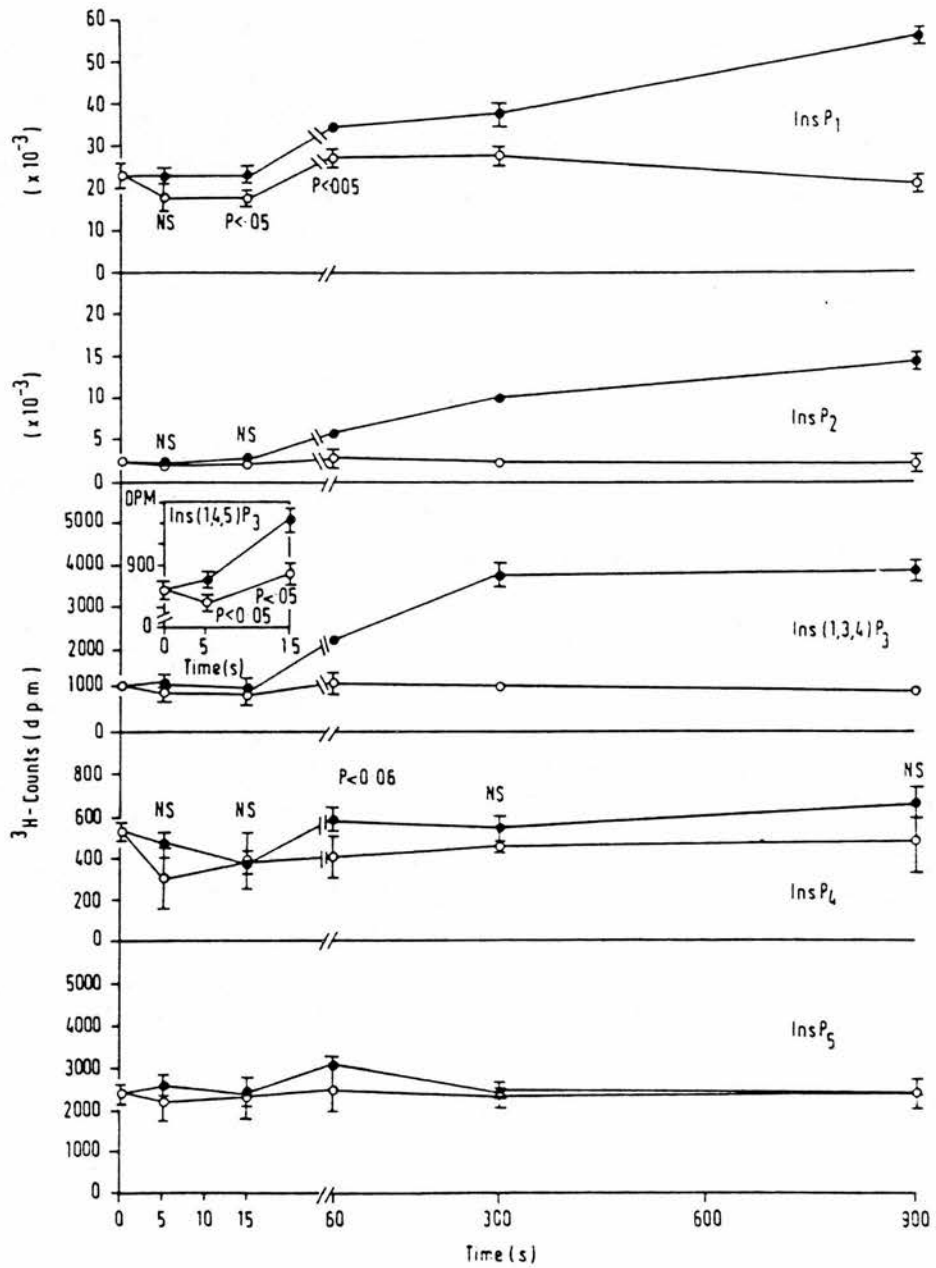


Fig 9: The effects of different antagonists on AcCh-stimulated phosphoinositol formation.

[^{3}H]Inositol-prelabelled cells were incubated, in the presence of Li^+ (10mM), with the antagonists shown and with or without AcCh (10^{-4}M) for 15 min. The radioactivity accumulating in the total phosphoinositol fraction of these cells is shown. Abbreviations and concentrations used are as follows: Nicotine 10^{-4}M (Nic), McNeil-A-343 10^{-4}M (McN), Atropine 10^{-6}M (Atr), Tubocurarine 10^{-6}M (Tub), Hexahydro-sila-difenidol 10^{-6}M (Hex), Glycopyrrolate 10^{-6}M (Gly), Propranolol 10^{-6}M (Prop) and Pirenzipine 10^{-5}M (Pir). Results shown are the mean±S.D. of values from triplicate incubations in a single experiment.

labelling of membrane phosphoinositides, but no definitive evidence for an activation of phosphoinositidase C in bovine zfr cells (11).

Fig. 9 summarises the ^{3}H-inositol labelled head group response to AcCh (10^{-4}M) and nicotine (10^{-4}M). The


Ins P1
Ins P2
Ins (1,3,4) P3
Ins P4
Ins P5
Ins (1,4,5) P3
DPM
900
0
0 5 15
Time (s)
P<0 05
P<0 005
P<0 06
NS
(x 10-3)
3H- Counts (d p m)
0 5 10 15 60 300 600 900
Time (s)

effect of a number of muscarinic antagonists and the nicotinic antagonist, tubocurarine, on the AcCh-stimulated head group response is also shown. Nicotine has no effect on the head group response, neither is the AcCh response blocked by tubocurarine. In contrast, the non-specific muscarinic antagonists, atropine and glycopyrrolate, completely antagonise the effects of AcCh. The relative lack of effect of pirenzipine, an M1 antagonist, and the antagonistic properties of hexahydro-sila-difenidol on the head group response are also illustrated.

Fig. 10 illustrates the time-course of appearance of ^{3}H-labelled phosphoinositol species in response to stimulation by AcCh (10^{-4}M) from cells pre-labelled with

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Fig 10 Time-course of appearance of [^{3}H]-labelled phosphoinositol species in response to AcCh.

[^{3}H]Inositol pre-labelled cells were exposed (●) or not exposed (o) to AcCh (10^{-4} M) in the presence of Li^{+} (10 mM), terminating each incubation over the time period 0 to 900s. The water-soluble extracts at each time-point were subject to HPLC analysis on a partisphere SAX-5 column, and the [^{3}H] counts in the peaks, identified (using appropriate standards) as InsP, $InsP_2$, $Ins(1,4,5)P_3$, $Ins(1,3,4)P_3$ and $InsP_4$ and $InsP_5$, separately measured. The figure shows the time-course of appearance of ^{3}H-counts in these different phosphoinositols species. Each point is the mean±S.D. of 3 HPLC analyses from 3 separate wells. The earliest time-point at which the stimulated cells differ significantly from the unstimulated cells for each phosphoinositol species is shown. Changes in labelling of $Ins(1,4,5)P_3$ at 5 s and 15 s are shown in the inset graph with the expanded scale.

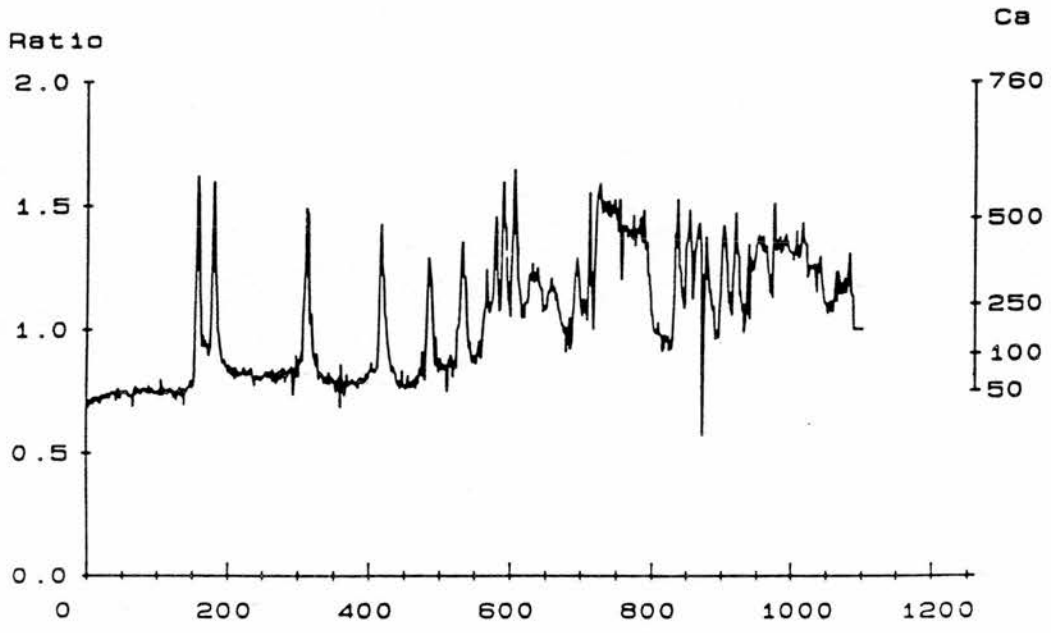

Fig 11 AcCh-stimulates oscillations in intracellular Ca^{2+} in single cultured bovine adrenal zona fasciculata/-reticularis cells.

Cells prepared on coverslips and pre-loaded with Fura 2, as described in Methods, were perfused with Krebs-Ringer/Hepes buffer alone and then with the same buffer containing AcCh (10^{-5}M). Changes in Fura 2 fluorescence in a single cell, reflecting changes in cytosolic [Ca^{2+}], were monitored as described. AcCh promoted a rapid rise in [Ca^{2+}] and sustained oscillations with an interspike interval of 18-120 sec. Individual spikes were of amplitude 300-550 nM from a resting level of 75 nM.

^{3}H-inositol to steady state. It is evident from this figure that an increase in labelling of $InsP_1$, $InsP_2$ and $InsP_3$ species is stimulated by AcCh. More detailed examination establishes that the earliest labelled species to show this increase is Ins(1,4,5)P_3, consistent with the activation by AcCh of a phosphoinositidase C specific for PtdIns(4,5)P_2.

The effect of AcCh on intracellular [Ca^{2+}]

Earlier work on the intracellular Ca^{2+} response to AcCh in cell populations of zfr cells (suspension culture) (12) was extended to a study of the intracellular Ca^{2+} response from single fura-2 loaded cells (in monolayer), using fluorescence enhancement microscopy. In Fig. 11 it can be seen that, in the case of the single, representative cell shown, AcCh leads to an oscillation in intracellular Ca^{2+}, sustained in the presence of normal extracellular [Ca^{2+}].

Discussion

Evidence presented here and elsewhere firmly establishes that cultured bovine zfr cells secrete cortisol in response to adrenergic and cholinergic stimulation (6,7,9,10,12). What is the significance of these responses in vivo?

In the case of the adrenergic response, a key question is whether or not the β_1 receptor is acquired as a result of the cell culture process itself (since the response is absent in freshly isolated cells). In contrast, the cholinergic response is clearly present in freshly isolated cells, arguing strongly for the potential to respond in vivo.

The demonstration that the adrenergic response is subject to homologous desensitisation with a $t_{0.5}$ of

approximately 2 h could provide a possible explanation for the absent cortisol response to adrenaline in fresh cells. Homologous desensitisation of β-adrenergic responses are common and often associated with short-term conformational changes in the receptor, followed by a longer-term degradation of the receptor (20). The possibility arises, therefore, that diffusion of catecholamines from medulla to cortex after death may desensitise the receptor in the freshly isolated cells; certainly evidence exists for the presence of catecholamines in the adrenal cortex after death (21). An absent response might also be the result of receptor degradation or damage during the isolation procedure itself.

The results reported here and elsewhere (15) conclusively establish that the adrenergic response in the zfr cells occurs through a β_1-receptor. This is of more than passing interest if Ungar's generalisation, that β_1-adrenergic receptors are associated with adrenergic nerve endings, is correct (22). It implies that any potential in vivo function for the adrenergic response depends upon adrenergic nerve stimulation.

Is there any evidence for adrenergic innervation of the mammalian adrenal cortex? Although there is no information on this question for the bovine cortex, evidence for adrenergic innervation of the adrenal cortex

exists in man (23), mouse (24) and rat (25). Hence, circumstantial evidence from receptor subclassification and from morphological studies suggests that any in vivo adrenergic effects may depend upon nerve stimulation. Even if this conclusion is correct - and it must still remain an open question - it begs several important questions (vide infra).

Several studies now firmly establish that bovine zfr cells also secrete cortisol in response to AcCh and that this depends upon AcCh binding to a muscarinic receptor (9,10,12). The muscarinic receptor has been shown by both pharmacological and molecular biological techniques to be heterogeneous in a variety of tissues. A range of muscarinic antagonists have established at least 3 types of muscarinic receptor subtype; the M_1 and the M_3 receptors are linked through a G-protein to the activation of phosphoinositidase C, whilst the M_2 receptor negatively couples to adenylate cyclase (26).

In this article and elsewhere (12), we establish that AcCh activates a phosphoinositidase C in bovine zfr cells, and that the release of Ins(1,4,5)P_3 brings about the expected increase in intracellular [Ca^{2+}]. Similar observations have been previously reported for the AcCh-stimulated release of aldosterone from bovine zg cells (27). The activation of phosphoinositidase C and the pharmacological evidence, reported here and elsewhere

(12), are consistent with AcCh's stimulation of cortisol secretion in bovine zfr cells occurring through its binding to an M_3 receptor. The significance of this classification to any possible in vivo effects is not yet clear.

That cholinergic stimulation may influence adrenocortical function in vivo is attested to by the morphological evidence for cholinergic innervation in man, cattle (28) and sheep (29).

These observations on the cholinergic response in bovine zfr cells lead to the same range of, as yet, unanswered questions as is the case for the adrenergic response. Why have a separate innervative control of steroidogenesis? How do adrenergic or cholinergic stimulation affect the steroid response of the cells to humoural factors such as ACTH or AII? How do the adrenergic and cholinergic mechanisms interact?

One possible explanation for separate innervative control relates to the increasing number of reports that bovine (and rat) zfr cells also respond to agonists which stimulate zg cells (e.g. AII and vasopressin, as well as AcCh and adrenaline) (30). Innervative control of different zones might allow targeting of a response to a specific zone of the adrenal cortex in a way which cannot be achieved by a circulating hormone. Adrenergic or cholinergic innervative stimulation might allow such

direct control, or a means of altering the relative proportions of different steroids secreted in response to other agonists through effects on the steroid pathway itself. The alternative possibility that such innervative responses may influence phenomena such as cell growth, however, must not be overlooked.

Whatever the specific answers to these questions, it is becoming increasingly clear that the possible involvement of both adrenergic and cholinergic innervative control cannot be overlooked in attempting to understand the control of adrenocortical function in vivo.

Finally, the observation that adrenaline and AcCh utilise different second messenger systems allows us to explore further the link between the second messengers formed, and the subsequent cellular responses. For AcCh, in particular, it should be possible to explore specifically the significance of intracellular, oscillatory $[Ca^{2+}]$ events in the function of this neurotransmitter in the adrenal cortex. Evidence that AcCh and AII mobilise a common intracellular pool of Ca^{2+}, presumably through $Ins(1,4,5)P_3$ formation already exists for populations of bovine zfr cells (31). This area can now be explored in more detail in single cells using fluorescence microscopy techniques.

Acknowledgements

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Further characterization of the steroidogenic responsiveness of purified zona fasciculata/reticularis cells from bovine adrenal cortex before and after primary culture: changing responsiveness to phosphoinositidase C agonists

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ABSTRACT

When bovine adrenocortical cells from the zona fasciculata/reticularis (zfr) are maintained in primary culture, cortisol secretion in response to acute stimulation with ACTH and adrenaline (which activate adenylate cyclase) is seen to increase steadily over the first 48 h, while secretion in response to angiotensin II and acetylcholine (which activate phosphoinositidase C) shows an initial decline in the first 24 h and a recovery to maximum after 48 h. We have investigated whether these discrepant changes in cortisol secretory response to the different agonists are due to changes in formation of the associated second messengers (cAMP or inositol phosphates), or altered coupling of these second messenger signals to steroid secretion.

Increases in steroid secretion in response to ACTH and adrenaline were paralleled by increased cAMP. Steroid secretion in response to exogenous 8-bromoadenosine 3′:5′-cyclic monophosphate also increased steadily during this 48-h period. Thus increased responsiveness was due to both increased second messenger formation and increased coupling to the steroid secretory response.

The decreased steroid secretory response to angiotensin and acetylcholine after 24 h, and subsequent recovery after 48 h in culture, were accompanied by an increased formation of phosphoinositols after 24 h and a further increase by 48 h. However, the steroid secretory response to a combination of calcium ionophore and the protein kinase C activator, phorbol 12-myristate 13-acetate, was reduced after 24 h and recovered by 48 h of culture. Fura-2-loaded cells also showed an increase in intracellular [Ca^{2+}] after 24 h in culture. Thus the impaired steroid secretory response to angiotensin II and acetylcholine after 24 h of culture was not due to reduced formation of second messengers but to a failure of Ca^{2+} and diacylglycerol so formed to activate the steroid secretory process.

Reversible uncoupling of the steroid secretory response from the Ca^{2+}- and diacylglycerol-based but not the cAMP-based second messengers observed in bovine zfr cells suggests that differential control of steroid secretion and other cell functions may be possible *in vivo* for activators of phosphoinositidase C, and may explain apparently discrepant results from studies on other in-vitro adrenocortical cell preparations.

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INTRODUCTION

The ability to maintain homogeneously dispersed cells from the rat (O'Hare & Neville, 1973) or bovine (Hornsby & Gill, 1978) adrenal cortex in primary culture has provided a valuable in-vitro model system for the study of the mechanisms which maintain adrenocortical structure and regulate its function. Thus, fibroblast growth factor has been found to be important in promoting adrenocortical cell division to confluence, and adrenocorticotrophin (ACTH) has been found necessary for maintenance of the steroid pathway for periods longer than a few days in primary culture (Hornsby & Gill, 1978).

In recent years, cultured cell preparations have been increasingly used to investigate the acute second messenger and steroid secretory responses to steroidogenic agonists. When plated at near confluence in a

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basic growth medium of nutrients/10% fetal calf serum/antibiotics/antifungal agents, bovine adrenocortical cells from the zona fasciculáta/reticularis (zfr) have consistently been reported to show increased responsiveness to ACTH compared with freshly isolated cells. Whether considered as steroid output/mg protein or as *n*-fold over basal output, the response is maintained or higher after 24 h and increases to a maximum by 48–72 h (Kawamura, Nakamichi, Imagawa *et al.* 1984; Williams, Lightly, Ross *et al.* 1989; Walker, Lightly, Clyne *et al.* 1991), declining thereafter.

We have previously reported that bovine adrenocortical zfr cells also secrete steroids in response to adrenergic agonists acting via a β_1 receptor (Lightly, Walker, Bird & Williams, 1990) coupled to adenylate cyclase (Kawamura *et al.* 1984; Walker, Lightly, Milner & Williams, 1988; Williams *et al.* 1989). The poor or absent steroidogenic response of freshly isolated cells to adrenergic agonists is followed by a markedly increased response over the first 48 h of primary culture. The increased steroid secretory response to both adrenaline and ACTH is paralleled by increased formation of adenosine 3′,5′-cyclic monophosphate (cAMP) (Kawamura *et al.* 1984; Walker *et al.* 1988).

Whilst investigating optimal growth conditions for studying the cholinergic response in bovine zfr cells, preliminary evidence was obtained that the cortisol response to acetylcholine was reduced at 24 h, compared with freshly isolated cells (Walker, Strachan, Lightly *et al.* 1990). It appeared that this fall in cortisol secretion at 24 h might also occur with angiotensin II (AII) (Walker *et al.* 1991). Since previous studies, discussed above, had suggested that the cortisol response to ACTH and adrenaline is increasing at 24 h, it was decided to undertake a detailed comparison of the day-by-day cortisol secretory response to ACTH/adrenaline, on the one hand, and AII/acetylcholine, on the other.

Since an obvious difference between ACTH and adrenaline versus AII and acetylcholine lies in the different second messenger responses promoted by these agonists, it was also decided to investigate changes in the second messenger responsiveness on a day-by-day basis. Unlike ACTH and adrenaline, which activate adenylate cyclase, both AII and acetylcholine stimulate a polyphosphoinositide-specific phospholipase C (phosphoinositidase C) to produce inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and diacylglycerol (DG), without increased cAMP formation (Bird, Meikle, Williams & Walker, 1989; Walker *et al.* 1990). The $Ins(1,4,5)P_3$ so formed liberates Ca^{2+} from intracellular stores which, combined with increased Ca^{2+} influx across the plasma membrane, leads to a sustained increase in cytosolic intracellular $[Ca^{2+}]$. This response, together with the activation of protein kinase C by DG, leads to increased steroid secretion. The activation of the steroid secretory response by Ca^{2+} and DG, independently of cAMP can also be successfully mimicked in these cells by the combined action of the Ca^{2+} ionophore A23187 and the phorbol ester phorbol 12-myristate 13-acetate (PMA) (Bird, Walker & Williams, 1990*b*).

In this paper we demonstrate that bovine zfr cells show a consistent fall in cortisol secretion at 24 h in culture to both AII and acetylcholine. This fall is shown to occur at a time when the membrane phosphoinositide turnover is increasing in response to these agonists. By studying the cellular responses to Ca^{2+} ionophore and phorbol ester, alone and in combination, together with measurements of intracellular $[Ca^{2+}]$, the probable site of the lesion responsible for this uncoupling is suggested. The implications of these findings are also discussed.

MATERIALS AND METHODS

Unless otherwise stated all materials were obtained as described by Bird, Nicol, Walker & Williams (1990*a*). Digitonin, A23187 and PMA were from Sigma Chemical Company, Poole, Dorset, U.K. Pyruvate and NADH were from the Boehringer Corporation, London, U.K. Sequential multi-channel analyser and computer (SMAC) chemistry calibrator 2 (SETpoint 2) standard (lyophilized bovine serum with added lactate dehydrogenase from chicken heart) was from Technicon Diagnostics, Basingstoke, Hants, U.K.

Cell preparation and culture

Cells were isolated and purified from the zfr of the adrenal gland obtained from 1- to 2-year-old steers, as described previously by Williams *et al.* (1989). Cells were used immediately for studies on freshly isolated cells, or cultured in 12-well plates (330 000 cells/ml per 2·25 cm well) in a medium of Ham's F10 with 10% controlled process serum replacement (CPSR 5; Sigma), penicillin (50 IU/ml), streptomycin (50 μg/ml) and amphotericin B (2·5 μg/ml) (F10 growth medium). After 24 h, medium was removed and replaced with fresh medium (0·5 ml/well).

Preparation of agonists

AII, ACTH, acetylcholine, adrenaline and 8-bromoadenosine 3′:5′-cyclic monophosphate (8-BrcAMP) were prepared by dilution in the media described below as required. A23187 and PMA were initially dissolved in dimethyl sulphoxide (DMSO) to prepare a stock solution of 10 mmol/l. This was then diluted 1 : 500 with the appropriate medium to give a 20 μmol/l solution in 0·2% (w/v) DMSO. Addition of this to cell incubations at 50 μl (25 μl A23187 solution

and 25 µl PMA solution) per 500 µl final volume gave a final concentration of 1 µmol/l in 0·02% DMSO. Parallel preparations of DMSO alone in medium were tested in each experiment but no significant effects of these DMSO controls were seen on steroid production over controls without DMSO.

Stimulation of cortisol and cAMP secretion

The basic protocol for experiments on freshly isolated cells (in suspension) or for plated cells (maintained in culture) was the same. Medium changes on cells in suspension were carried out by first pelleting the cells (centrifugation in a microfuge at 500 *g* for 3 min) and then resuspending in fresh medium. For cultured cells, the well contents were removed by aspiration and immediately replaced with fresh medium. All incubations were subsequently carried out in a gassed, humidified incubator (5% CO_2 at 37 °C).

Cells were washed twice with 1 ml Earle's balanced salts (EBS) solution and finally resuspended in EBS with added bovine serum albumin (BSA) and glucose (0·2 and 0·1% (w/v) respectively) (EBSBG; 0·45 ml/well). After 5 min preincubation at 37 °C, agonists were added in 50 µl EBSBG and cells incubated for a further 60 min. At the end of this time cell-free medium was recovered (by centrifugation of cell suspensions or aspiration of wells) and stored at −20 °C for cortisol radioimmunoassay, as described previously (Williams *et al.* 1989).

Where cAMP secretion into the medium was also measured, an identical protocol was followed but the final incubation volume in each well was 1 ml. At the end of the experiment the medium was recovered and split into two 0·5 ml volumes. Cortisol content of the recovered medium was assayed directly as above, while cAMP content was determined by radioimmunoassay after prior acetylation of 0·5 ml of the recovered medium (Williams *et al.* 1989).

Stimulation of phosphoinositidase C response

The activation of phosphoinositidase C by agonists was determined by measuring the hormone-stimulated generation of [^{3}H]inositol phosphates in the presence of 10 mmol Li^+/l.

Labelling of cultured cells with [^{3}H]inositol was first achieved by supplementing the F10 growth medium in each well with [^{3}H]inositol to 10 µCi/ml and incubating for 5 h. Freshly isolated cells were labelled for the same period of time after resuspension in growth medium (at 300 000/0·5 ml) with [^{3}H]inositol (10 µCi/ml), and dispensing to microfuge tubes at 0·5 ml/tube.

Once labelled, the [^{3}H]inositol-containing F10 growth medium was removed and replaced with EBSBG (0·5 ml/well or tube) and cells were incubated for 15 min. Medium was replaced with EBSBG–10 mmol LiCl/l–10 mmol inositol/l (EBSBG/Li^+/Ins; 0·45 ml/well or tube) and cells were incubated for 15 min. Agonists were then added in a total of 50 µl EBSBG/Li^+/Ins and cells incubated for a further 15 min. Cell stimulation was finally terminated by the addition of ice-cold perchloric acid (PCA; 250 µl, 15%). For cultured cells, wells were scraped and the contents recovered to microfuge tubes with a 0·5 ml water wash. For cells in suspension, 0·5 ml water was added to each tube after addition of the PCA. The water-soluble [^{3}H]inositol phosphates were then recovered by pelleting insoluble material (centrifugation at 3300 *g* for 3 min) and retaining the supernatant. These aqueous extracts were neutralized by thorough mixing with 1·5 ml freon–octylamine (1,1,2-trichlorotrifluoroethane–tri-n-octylamine; 1 : 1) and the resulting three phases separated by brief centrifugation in a bench centrifuge. Neutral aqueous extracts (top phase) were recovered and stored frozen at −20 °C for assay of [^{3}H]inositol phosphates.

Assay of [^{3}H]inositol phosphates

The [^{3}H]inositol phosphates in aqueous extracts were separated from the [^{3}H]inositol by retention of the former on columns of anion exchange resin (analytical grade 1-X8, 100–200 mesh) as described previously (Bird *et al.* 1990*a*). Combined [^{3}H]inositol phosphates were recovered by elution with 1 mol ammonium formate/l–0·1 mol formic acid/l and radioactivity was determined by liquid scintillation counting in a Packard 1900CA liquid scintillation counter.

Protein determination

Cellular protein was measured each day by washing cells three times in EBS (0·5 ml) and solubilizing in 1% Triton X (0·5 ml/well or tube, incubation at 37 °C for 15 min followed by scraping of cells with a rubber spatula). Samples were stored frozen (−20 °C) before dilution at 1 : 10 with water and assay by the Bradford method, as adapted for the Cobas Fara centrifugal analyser (see Williams *et al.* 1989).

Cellular lactate dehydrogenase release and assay

Cells were washed and resuspended in intracellular buffer (0·45 ml; composition: KCl (120 mmol/l), NaCl (10 mmol/l), $MgSO_4$ (4·18 mmol/l), EGTA (1 mmol/l), Hepes (20 mmol/l); pH 7·4) and permeabilized by addition of digitonin (final concentration 50 µmol/l in 500 µl final volume). Permeabilization was allowed to proceed at 37 °C for 15 min. A cell-free supernatant was then obtained by removal of medium and centrifugation at 3000 *g* (3 min). Samples were stored (as two × 200 µl volumes) at 4 °C for assay in duplicate.

Samples were completely stable at 4 °C for several days but showed reduced activity when frozen.

The assay procedure used was a modification of the method of Brydon & Smith (1973), adapted for the Cobas Fara centrifugal analyser. This assay monitors the change of NADH to NAD as pyruvate is converted to lactate. The method described here was carried out in the absence of urea, so total (i.e. urea stable and unstable) lactate dehydrogenase was measured.

Stock NADH (0·2 mmol/l) solution and stock sodium pyruvate (7·7 mmol/l) solutions were prepared in 0·1 mol KH_2PO_4/l (buffered to pH 7·4 with NaOH) and stored at −20 °C (stable for several weeks). For assay of lactate dehydrogenase (LDH) activity, 200 µl NADH solution and 10 µl sample in each rotor cuvette were prewarmed to 37 °C and blank absorbance values read at 340 nm. Stock pyruvate solution (25 µl) was added to start the reaction and, from 6 s after the start, absorbance changes were measured at 10-s intervals for 100 s. With diluent additions the total assay volume was 275 µl and final concentrations of NADH and pyruvate were 0·144 and 0·7 mmol/l respectively. LDH activity was determined from the initial rate of decrease of absorbance and expressed as LDH activity units (applying a correction factor of 1600). All assays were carried out with a quality control standard of activity 50–60 U/l (SETpoint 2 standard diluted ten times with intracellular buffer). For this quality control standard, the variation of values obtained in six separate assays on 6 consecutive days was 58·6 ± 3·1 U/l (mean ± S.D., range 53–62).

Measurement of intracellular calcium

Cells purified as described above were plated onto 22 mm glass coverslips placed in 12 multiwell plates. After 24 h in culture, cells were loaded with fura-2 AM (5 µmol/l) for 30–60 min at 37 °C. A coverslip with loaded cells was then placed in a thermostated superfusion chamber attached to the stage of a Nikon diaphot inverted microscope. Cells were perfused at 37 °C with a modified Krebs–Ringer medium containing NaCl (134 mmol/l), KCl (4·5 mmol/l), $MgCl_2$ (1 mmol/l), NaH_2PO_4 (1 mmol/l), $CaCl_2$ (1·5 mmol/l), glucose (5·5 mmol/l) and Hepes (10 mmol/l) pH 7·4, and excited alternately at 340 and 380 nm. Fluorescence emissions at the two wavelengths were recorded at 506 nm and stored separately on a microcomputer. The fluorescence ratio ($F_{340/380}$) was plotted as a function of time (after correcting for background counts) using a dedicated Amstrad 2086 microcomputer.

Statistical analysis

Significance was established by comparison of data using Student's *t*-test.

RESULTS

The changes in cortisol secretion and cAMP secretion observed in response to ACTH (0·1 nmol/l, 1 h) or adrenaline (1 µmol/l, 1 h) on maintenance of bovine zfr cells in primary culture are shown in Fig. 1. All data are expressed as *n*-fold over basal (to allow comparison with data in Fig. 2) but basal values are given in the figure legend. For comparison, the cortisol secretory response to 8BrcAMP (1 mmol/l, 1 h) is also shown.

Freshly isolated cells secreted cortisol in response to ACTH but not adrenaline; at 24 h in culture the cells also responded to adrenaline. An increase in cortisol secretory response to both ACTH and adrenaline was observed over a 48-h period, continuing, in this case, to a maximum at 72 h of culture (day 4) but declining thereafter. The cortisol secretory response to ACTH and adrenaline was significantly higher on each day than that on the previous day ($P<0·01$ in each case, from day 1 to day 4). This increased cortisol secretory response was paralleled by a corresponding increase in cAMP formation in each case (Fig. 1).

Freshly isolated cells also secreted cortisol in response to 8BrcAMP, and this response increased steadily to a maximum over the first 48–72 h of culture, after which it too declined (ACTH: cAMP secretion significantly increased from days 1 to 2 ($P<0·05$) and days 2 to 3 ($P<0·01$) but not days 3 to 4; adrenaline: cAMP secretion not significantly increased from days 1 to 2 but significantly increased from days 2 to 3 ($P<0·01$) and days 3 to 4 ($P<0·01$)).

The changes in AII- and acetylcholine-stimulated cortisol secretion and phosphoinositidase C activation on each day of primary culture are shown in Fig. 2. All results are expressed as *n*-fold over basal response (with basal values given in the figure legend) to allow data to be combined from several experiments.

While all the results shown are expressed as *n*-fold, the raw data also showed similar trends, and the protein content of each well, taken as a measure of cell number, did not vary by more than 20% over the 5-day period. As an independent check of cell number on each day, lactate dehydrogenase activity was released from cells (permeabilized with digitonin) and assayed as described. The results obtained agreed closely with the results from protein determinations (results not shown).

Figure 2 shows that, after 24 h in culture (day 2), the cortisol response to AII and acetylcholine was reduced (AII: $P<0·01$ in three experiments; acetylcholine: $P<0·05$ in two experiments or unchanged (one experiment)) but rose once more to a maximum after a total of 48 h in culture (day 3) (Fig. 2). The response thereafter declined (cortisol secretion on day

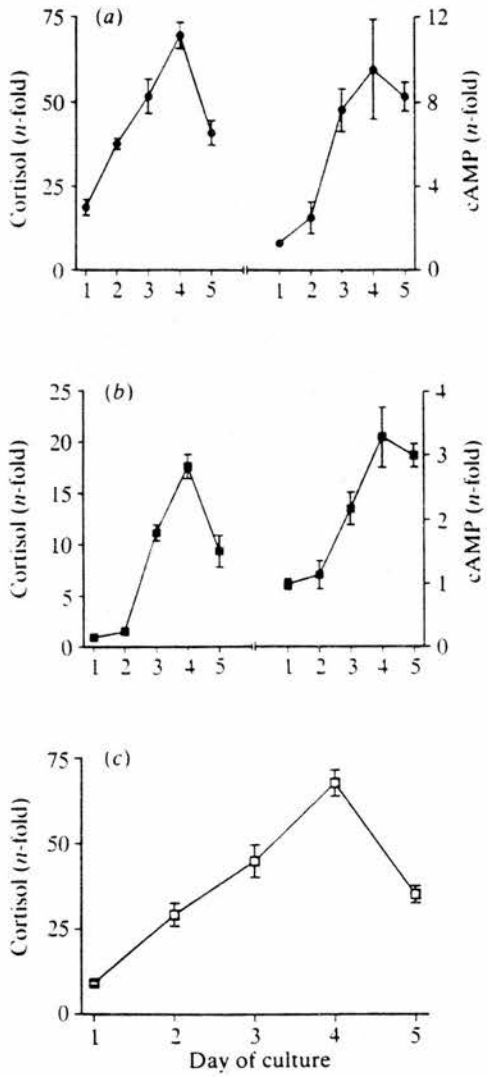

FIGURE 1. Cortisol and cAMP secretory responses of bovine zona fasciculata/reticularis cells stimulated for 1 h with the agonists (*a*) ACTH(1–24) (0·1 nmol/l) or (*b*) adrenaline (1 μmol/l) on the day of isolation (day 1) and on successive days after maintenance in primary culture (from experimental days 2 to 5). (*c*) Cortisol response to 8-bromoadenosine 3′:5′-cyclic monophosphate (1 mmol/l). For experimental details see Materials and Methods. All results are expressed as *n*-fold over basal (unstimulated) response and show the mean ± S.D. of triplicate wells from a representative experiment performed on three different occasions with different cell preparations. Mean basal values for cortisol secretion were 6·80, 7·84, 13·10, 11·76 and 15·12 pmol/well per h per 100 g protein for days 1 to 5 respectively, and mean basal cAMP secretion was 0·129, 0·234, 0·228, 0·176 and 0·190 pmol/well per h per 100 g protein respectively.

4 being less than on day 3; $P<0·01$ for AII and $P<0·05$ for acetylcholine in all three experiments).

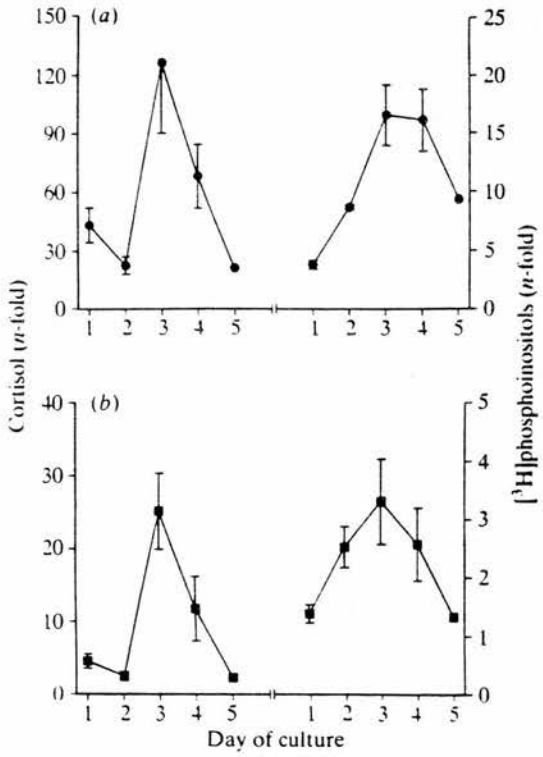

FIGURE 2. Cortisol secretory response and phosphoinositidase C response (generation of [^{3}H]phosphoinositols) of bovine zona fasciculata/reticularis cells stimulated with (*a*) angiotensin II or (*b*) acetylcholine shown on the day of isolation (day 1) and on successive days after maintenance in primary culture (from experimental days 2 to 5). See Materials and Methods for experimental details. All results are expressed as *n*-fold over basal (unstimulated) response and show the mean ± S.E.M. of mean values from three separate experiments, each performed using triplicate wells for each data point. Mean basal values for cortisol secretion were 6·40, 1·25, 3·25, 5·60 and 2·75 pmol/well per h per 100 g protein for days 1 to 5 respectively, and mean basal [^{3}H]inositol phosphates labelling were 345, 657, 723, 346 and 321 d.p.m./100 g protein respectively.

The effect of AII and acetylcholine on phosphoinositidase C activation in these cells did not parallel the day-by-day changes in steroid secretory response. Both agonists promoted a greater formation of [^{3}H]inositol phosphates after 24 h in culture (day 2, increase over day 1 significant for AII ($P<0·01$) in all three experiments and acetylcholine ($P<0·01$) in two out of three experiments) at a time when there is a marked reduction in the cortisol secretory response. The [^{3}H]inositol phosphate responses to AII and acetylcholine then rose to a maximum after 48 h in culture (day 3), the response on day 4 being significantly ($P<0·01$) less in two out of three experiments for both AII and acetylcholine.

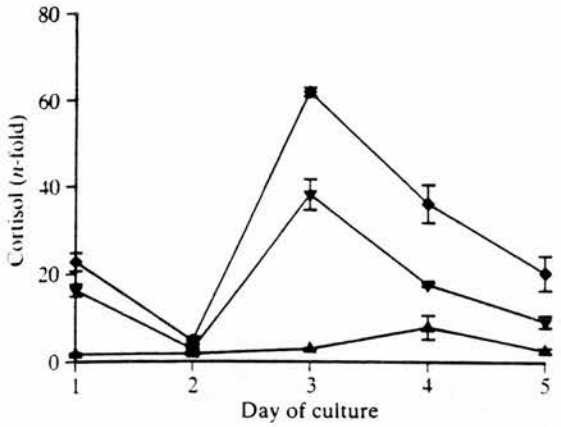

FIGURE 3. Cortisol secretory response to A23187 alone (▲), phorbol 12-myristate 13-acetate (PMA) alone (▼) or A23187 and PMA combined (◆) is shown for freshly isolated bovine zona fasciculata/reticularis cells (day 1) and for successive days in primary culture (experimental days 2 to 5). For experimental details see Materials and Methods. All results are expressed as *n*-fold over basal (unstimulated) response and show the means ± S.E.M. for triplicate wells in a representative experiment. Qualitatively identical patterns were obtained in a total of five experiments with A23187 and PMA combined, and in two experiments with A23187 and PMA alone.

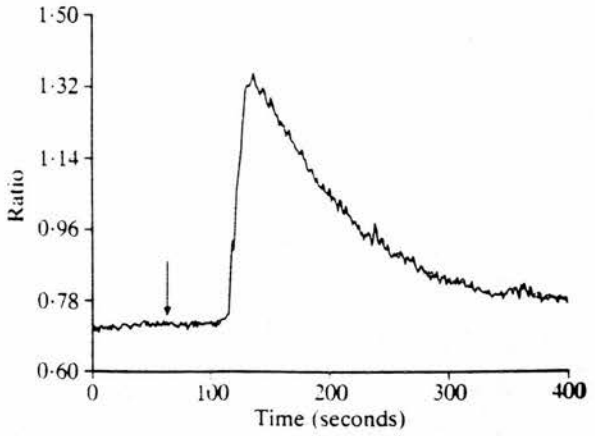

FIGURE 4. Intracellular [Ca^{2+}] response to angiotensin II (AII) (0·1 μmol/l) from a single fura-2-loaded bovine zona fasciculata/reticularis cell at 24 h in culture (day 2). The chamber was continuously perfused with modified Krebs buffer (see Materials and Methods) at 37 °C, monitoring the ratio of fluorescence emission at 506 nm following alternate excitation at 340 and 380 nm (F_{340}/F_{380}) versus time (s). AII (0·1 μmol/l) was infused in the same Krebs buffer (arrow). A representative cell is shown in which the increase in the ratio (F_{340}/F_{380}) in response to the AII infusion is shown. A total of 40 cells from five different cell preparations was examined in this way; a rise in intracellular [Ca^{2+}] was observed in 68% cells in response to AII.

Figure 3 shows a representative experiment of the effects of A23187 alone, PMA alone and A23187 and PMA combined on the cortisol secretory response. The initial changes in cortisol secretory response to PMA/A23187 were qualitatively similar to the changes described for AII and acetylcholine. After 24 h in culture (day 2) the response was also much reduced ($P<0·05$ in each of five experiments) but increased once more after 48 h in culture (day 3) ($P<0·05$ in all five experiments). In two experiments, the effects of A23187 and PMA alone were examined. Figure 3 shows that PMA alone was a much more effective stimulus to cortisol secretion than A23187 alone and that the day-by-day secretory pattern was very similar to the A23187/PMA combination, with a statistically significant fall in cortisol secretion at 24 h ($P<0·05$ in each of two experiments). Although A23187 alone was relatively ineffective, the steroid secretion at 24 h was unchanged, compared with the freshly isolated cells.

Figure 4 shows the increase in intracellular [Ca^{2+}] in response to AII (0·1 μmol/l) from a single representative cell after loading with fura-2 on day 2 of primary culture. Forty cells from five separate primary cell isolates were studied on day 2 by this method, with qualitatively similar responses observed in 68% of the cells examined, comparable with the findings for cells on day 3.

DISCUSSION

Data in Fig. 1 show that the increase in cortisol secretion to ACTH and adrenaline on each of the first 4 days in culture was closely paralleled by changes in cAMP formation. However, the increased cAMP formation cannot be the only explanation for the increased cortisol secretion since the steroid secretory response to 8BrcAMP also increased steadily over the first 72 h of culture. Thus, post-second messenger factors, such as increased availability of cAMP-dependent protein kinase or its substrates or increased steroid synthetic capacity must also be relevant.

In contrast, the cortisol secretory response to the agonists which activate phosphoinositidase C, AII and acetylcholine was consistently reduced by 24 h of culture (day 2) (Fig. 2), thereafter recovering. Measurement of the protein content of the wells confirmed that this fall was not due to any significant fall in cell number (results not shown). Accompanying this fall in cortisol secretion on day 2, there was a paradoxical increase in [^{3}H]inositol phosphate formation. Hence, the decline in cortisol secretion could not have been due to reduced activation of the polyphosphoinositide-specific phosphoinositidase C, whether through marked reductions in receptor

numbers or affinity, in G-protein availability or in cell phosphoinositidase C activity.

There are several explanations which might account for the dissociation between [^{3}H]inositol phosphate formation and cortisol secretion: (1) a requirement for additional diacylglycerol formed by the breakdown of phospholipids other than the phosphoinositides (through activation of an additional phospholipase C); (2) a failure to elevate intracellular [Ca^{2+}]; (3) a failure to couple the increased [Ca^{2+}] and/or DG signals to activation of the rate-limiting step of the steroid pathway cytochrome P_{450} side-chain cleavage enzyme; (4) a failure to convert the pregnenolone so formed into cortisol.

The first two possibilities appear to be unlikely when the effects of combined Ca^{2+} ionophore and PMA are considered. These agents induce an elevation of intracellular [Ca^{2+}] and activation of protein kinase C independently of the receptor/G protein/phosphoinositidase/Ca^{2+} channel systems, and yet failed to restore the markedly reduced cortisol secretory response in cells on day 2 (Fig. 3). Furthermore, studies using fura-2-loaded cells on day 2 confirmed that generation of the intracellular Ca^{2+} signal was unimpaired (Fig. 4). The fourth possibility can also be eliminated as no corresponding reduction in steroid secretion was seen in response to ACTH or 8BrcAMP on day 2. Also, day-by-day changes in corticosterone secretion paralleled changes in cortisol secretion for ACTH and AII respectively (E. R. T. Lightly, unpublished data).

These findings are therefore consistent with a failure to couple the Ca^{2+} and/or DG signals to activation of $P450_{scc}$. In view of the known involvement of Ca^{2+} in the cortisol secretory response to ACTH (which is maintained on day 2) it is unlikely that the fault lies in the coupling of the Ca^{2+} signal to activation of $P450_{scc}$. This is further supported by the experiments in which ionophore was studied alone; A23187 alone achieved a slight but significant stimulation of cortisol secretion, with no significant difference between days 1 and 2 (Fig. 3). In contrast, phorbol ester alone was a more potent stimulus to cortisol secretion, but was much less effective on day 2, compared with day 1 (Fig. 3). It seems most likely, therefore, that there is a failure of DG, formed by breakdown of the phosphoinositides, to activate protein kinase C in day-2 cells, either because of a loss of kinase C itself or the loss of one of its substrates.

If cells are cultured under the conditions described for longer than 24 h, a marked recovery of the cortisol secretory response to AII and acetylcholine is observed, reaching a maximum on day 3. The response to A23187/PMA also increases to a maximum on day 3, as does the phosphoinositidase C response to AII and acetylcholine. Whilst other factors may contribute to this maximum, it is evident that a recovery of the coupling of the second messenger responses to activation of $P450_{scc}$ must occur.

Stimulation of phosphoinositidase C activity without an accompanying steroid response has been observed *in vitro* in both isolated rat zfr cell preparations maintained for 3 days in culture before being stimulated with vasopressin (Gallo-Payet, Guillon, Balestre & Jard, 1986), and in Y-1 cells stimulated with vasopressin (Langlois, Arroub, Saez & Begeot, 1988) or AII (Langlois, Saez & Begeot, 1990). In the latter case, the cells also failed to respond to PMA, even though protein kinase C was demonstrated to be present. The failure in each case to observe a steroid response is at odds with the reported ability of vasopressin to stimulate corticosterone secretion by the zfr of perfused rat adrenal glands and superfused cells (Hinson, Vinson, Porter and Whitehouse, 1987).

More recently, it has been shown that cultured ovine adrenal fasciculata cells possess a similar number of high affinity AII receptors as bovine fasciculata cells. Whilst AII activated a phosphoinositidase C and increased intracellular [Ca^{2+}] in both cell types, it led to the stimulation of steroid secretion only from the bovine cells; ovine cells failed to secrete glucocorticoids (Viard, Rainey, Capponi *et al.* 1990). Clearly, the situation for the bovine fasciculata cells is more complicated than reported by Viard *et al.* (1990), since at 24 h these cells enter a state similar to that described for ovine fasciculata cells, but recover thereafter. Our findings do not rule out the possibility that ovine cells might also secrete steroid in response to AII, depending upon the time after initial plating when experiments are carried out.

An important consequence of our findings is that if cell cultures are to be used as an experimental model it is necessary to consider on which day to carry out the experiments. In the case of bovine zfr cells, experiments using phosphoinositidase C agonists on day 2 or after day 4 would give poor cortisol responses. A further implication is that the secretion of cortisol by adrenocortical zfr cells could perhaps be dissociated from the ability of AII and other phosphoinositidase C agonists to affect other cellular functions such as the stimulation of mitosis (Gill, Ill & Simonian, 1977). This possibility should be open to experimental testing in the culture of bovine zfr cells.

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STUDIES OF HORMONE-SENSITIVE AND -INSENSITIVE POOLS OF PHOSPHOINOSITIDES IN CULTURED BOVINE ZONA FASCICULATA/RETICULARIS CELLS

EVIDENCE THAT ACETYLCHOLINE AND ANGIOTENSIN II STIMULATE THE BREAKDOWN OF A COMMON POOL OF PHOSPHOINOSITIDES

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Abstract—The effects of acetylcholine (ACh) and manganese pre-incubation on angiotensin II (AII)-stimulated incorporation of [^{3}H]inositol into phosphoinositide, phosphoinositol and free inositol fractions of adrenocortical cells isolated from the bovine zona fasciculata/reticularis (zfr) were investigated. In cells pre-labelled for 6 hr with [^{3}H]inositol, ACh and AII stimulated the incorporation of cytosolic [^{3}H]inositol into a common hormone-sensitive pool of phosphoinositides, which was distinct from the non-hormone-sensitive pool labelled in the presence of manganese. Regression analysis of the cortisol versus [^{3}H]inositol headgroup responses for both AII (10^{-11}–10^{-7} M) and ACh (10^{-9}–10^{-3} M) showed that the gradients of these responses were not significantly different. These data provide strong evidence that in cultured bovine zfr cells, ACh and AII stimulate the breakdown and resynthesis of a common pool of phosphoinositides.

Contrary to the earlier view that adrenocorticotrophin (ACTH§) is the sole stimulus of steroid secretion from the inner zones of the adrenal cortex, it is now clear that zona fasciculata/reticularis (zfr) cells secrete steroids in response to a wide variety of hormones and neurotransmitters. Studies by ourselves and others have established that bovine zfr cells, either freshly isolated or in primary culture, secrete cortisol in response to catecholamines [1, 2], angiotensin II (AII) [2, 3], acetylcholine (ACh) [2, 4, 5] and vasopressin [6] in addition to ACTH. Of these agonists, AII and ACh have been shown to exert this effect through the activation of a hormone-sensitive phosphoinositidase C [6, 7]. Neither agonist has any acute effect on cAMP levels, whereas both promote the rapid and dose-dependent formation of water-soluble [^{3}H]phosphoinositols. The phosphoinositols formed and their time-course of appearance are consistent with the activation of a phosphoinositide-specific phosphoinositidase C. In fura-2-loaded zfr cell suspensions, both AII and ACh stimulate dose-dependent increases in cytosolic [Ca^{2+}], and both agonists appear to mobilize a common intracellular Ca^{2+} pool [8]. Furthermore, in single fura-2-loaded bovine adrenocortical cells, oscillations in intracellular [Ca^{2+}] have been observed in response to both agonists [9, 10].

It is also clear that individual cells can respond to both AII and ACh as both agonists mobilize a common intracellular Ca^{2+} pool. It is not clear, however, whether ACh and AII receptors are coupled to phosphoinositidase C which acts on separate subpools of phosphoinositides, or whether phosphoinositidase C acts on a common pool of phosphoinositides. This question is particularly relevant given the evidence for the existence of multiple pools of phosphoinositides in these and other cells [11–13].

In this paper we present evidence that AII and ACh act to stimulate both the breakdown and resynthesis of a common pool of phosphoinositides.

MATERIALS AND METHODS

Materials. Ham's F10 growth medium, Earl's balanced salt solution, glutamine and other cell culture materials were from Northumbria Biologicals (Cramlington, U.K.). Penicillin, streptomycin and fungizone were obtained from Flow Laboratories (Rickmansworth, U.K.) and collagenase from Lorne Diagnostics (Bury St. Edmunds, U.K.). Controlled Process Serum Replacement No. 5, glucose, ACh, atropine, inositol and cortisol were purchased from the Sigma Chemical Co. (Poole, U.K.) and bovine serum albumin (fraction V) was obtained from ICN Biomedical (High Wycombe, U.K.). AII (Asp^1-Val^5) was the MRC international standard from the National Institute for Biological Standards and Control. *myo*[^{3}H]Inositol and cortisol tracer [cortisol-3-(*O*-carboxymethyl) oximino-(2-[^{125}I]iodohistamine)] were supplied by Amersham Inter-

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§ Abbreviations: zfr, zona fasciculata/reticularis; AII, angiotensin II; ACh, acetylcholine; ACTH, adrenocorticotrophin; GM, growth medium; EBSBG, Earl's balanced salt solution containing bovine serum albumin (0.2%) and glucose (0.1%).

national (Amersham, U.K.). 1,1,2-Trichlorotrifluoro-ethane and tri-*n*-octlyamine were purchased from the Aldrich Chemical Co. (Gillingham, U.K.), and all other chemicals to Analar grade from BDH (Thornlibank, Glasgow, U.K.). AG1X8 anion exchange resin (100–200 mesh, formate form) was obtained from Bio Rad Laboratories (Watford, U.K.). Scintillation fluid (299) and scintillation vials were purchased from Canberra Packard (Pangbourne, U.K.), and scintillation counting was carried out on a Canberra Packard 1900CA liquid scintillation counter.

Cell culture. Bovine adrenal glands were obtained from freshly slaughtered 1–2-year-old steers at the local abattoir, and purified zfr cell suspensions prepared as described previously [3]. Cells were plated out at a density of 333,000 cells/well in multiwell plates (12 × 25-mm diameter wells) in 1 mL/well Ham's F10 medium supplemented with 10% Controlled Process Serum Replacement No. 5, penicillin (50 IU/mL), streptomycin (50 μg/mL) and amphotericin B (2.5 μg/mL) (growth medium, GM). GM was renewed after 24 hr (0.5 mL), and the cells maintained in primary culture for a further 48 hr before use. Labelling of the phosphinositides was achieved by inclusion of [^{3}H]inositol in the GM (10 μCi/mL, 0.5 mL) for the last 6 hr of culture (Figs 1 and 2) or for the entire 48-hr period (Fig. 3) where steady-state labelling was required [7].

Where manganese-stimulated labelling was investigated, $MnCl_2$ (1 mM) was included in the GM during the 6-hr labelling period. Where cells were pre-stimulated with ACh during labelling, 5 μL of a stock solution of ACh (in GM) was added to each well to a final concentration of 10^{-4} M for the last hour of labelling only. Pre-stimulation was terminated by addition of 5 μL of the ACh antagonist atropine (10^{-6} M final concentration) to each well for 5 min.

Agonist stimulation of phosphoinositidase C. All agonists were dissolved in Earl's balanced salt solution containing bovine serum albumin (0.2%) and glucose (0.1%) (EBSBG). After the labelling period, GM was replaced with EBSBG (0.5 mL/well), or EBSBG containing atropine (10^{-6} M) if cells had been stimulated previously with ACh. Cells were incubated for 15 min after which medium was replaced with EBSBG with added LiCl (10 mM) and inositol (10mM) (0.45 mL/well). After a further 15 min incubation, agonist solutions (50 μL) were added to a final volume of 0.5 mL, and stimulation allowed to continue for the times indicated before termination by addition of ice-cold perchloric acid (15% v/v, 250 μL/well).

Recovery and measurement of [^{3}H]*inositol-labelled products.* Following addition of perchloric acid to terminate cell stimulation, the base of each well was scraped using a rubber policeman and the contents transferred to an Eppendorf tube, with a 0.5 mL water wash. Following centrifugation (3300 *g* × 3 min), the acid supernatant was transferred to a glass tube and neutralized by mixing with a 1:1 mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoro-ethane (1.5 mL). Following a brief centrifugation (20 *g* × 5 min) to separate the phases, 0.9 mL of the upper aqueous phase was recovered and stored at −20° prior to assay.

For assay of [^{3}H]inositol and [^{3}H]phosphoinositols, samples were thawed, EDTA added to a final concentration of 1 mM and samples loaded onto individual columns of AG1X8 anion exchange resin (0.25 mL). The columns were washed with water (2 × 4 mL), and the combined eluates from loading and washing (containing free [^{3}H]inositol) collected into a 20 mL scintillation vial. Bound [^{3}H]inositol phosphates were then eluted directly into a fresh 20-mL vial with 1 M ammonium formate–0.1 M formic acid (2 × 2 mL). Following addition of scintillation fluid (10 mL), radioactivity of the samples was determined by liquid scintillation counting. All values shown are volume corrected, and were counted for 10 min each, or to an error of <1%.

The pelleted material recovered from the centrifuged perchloric acid extracts was frozen at −20° under 200 μL water. After thawing, the loosened pellets were broken up by vortexing and dissolved by addition of $CHCl_3$–MeOH–concnHCl (100:200:1 v/v) (0.75 mL), followed by $CHCl_3$ and HCl (0.1 M) (both 250 μL). The resulting aqueous and organic phases were separated by centrifugation (20 *g* × 5 min) and 400 μL of the lower phosphoinositide-containing phase recovered to a scintillation vial. The solvent was removed under air (Techne sample concentrator), 3 mL scintillation fluid added, and radioactivity determined by scintillation counting.

Agonist stimulation of cortisol secretion. For studies on cortisol secretion from unlabelled cells, cells were washed in EBSBG (2 × 1 mL) and incubated in EBSBG for 5 min (0.45 mL). Following addition of agonists to a final volume of 0.5 mL, cells were incubated for 1 hr after which the overlying medium was removed and stored at −20° prior to cortisol radioimmunoassay [14].

Statistical analysis. Statistical significance between results was assessed using Student's *t*-test. Values are given as the means ± SD, unless otherwise stated.

RESULTS

Figure 1 shows the results of studies carried out on cells pulse-labelled for 6 hr (not to steady state) with [^{3}H]inositol, in the presence or absence of ACh (10^{-4} M) or Mn^{2+} (10^{-3} M) as described, and subsequently challenged with AII (10^{-7} M) (in the presence of lithium) for 15 min. The distribution of radioactivity between phosphoinositide (a) free inositol (b) and total phosphoinositol fractions (c) is shown. Under these labelling conditions the bulk of the radiolabel is found in the free inositol fraction.

In control cells (no pre-stimulation during labelling), subsequent acute (15 min) stimulation with AII increased the turnover of the phosphoinositides as indicated by increased accumulation of label in both the phosphoinositides (a) and phosphoinositols (c), with a corresponding decrease in free [^{3}H]inositol (b) (all $P < 0.01$). Although labelling in the presence of ACh increased the incorporation of [^{3}H]inositol into the phosphoinositides ($P < 0.05$), the maximal incorporation

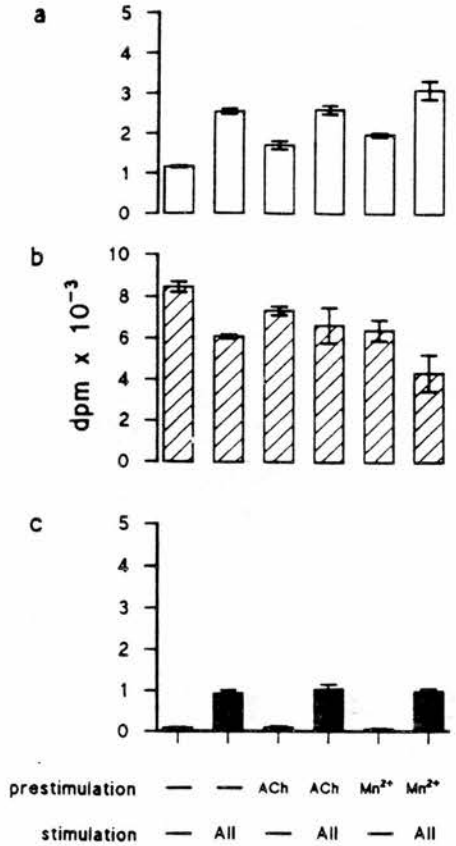

Fig. 1. Effect of ACh and Mn^{2+} pre-stimulation on subsequent acute AII-stimulated redistribution of radioactivity between [^{3}H]phosphoinositide, [^{3}H]inositol and [^{3}H]phosphoinositol fractions of pre-labelled bovine zfr cells. Cells were pre-labelled for 6 hr with [^{3}H]inositol in the presence or absence of MnCl (10^{-3} M) or ACh (10^{-4} M), as described. Cells were subsequently incubated for a further 15 min in the presence or absence of AII (10^{-7} M) in fresh medium containing 10 mM Li^+. The distribution of radioactivity between the [^{3}H]-phosphoinositide (panel a), [^{3}H]inositol (panel b) and [^{3}H]-phosphoinositol (panel c) fractions is shown. For further experimental details see Materials and Methods. Values shown are the means ± SD of triplicate determinations from one representative experiment of three similar experiments.

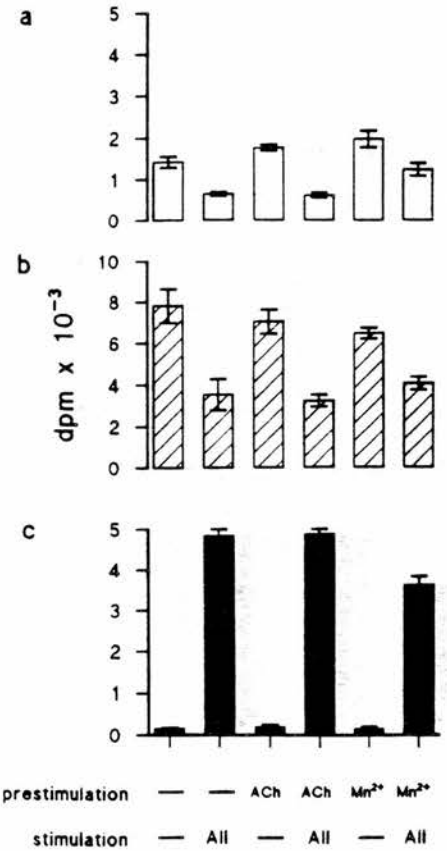

Fig. 2. Effect of ACh and Mn^{2+} pre-stimulation on subsequent chronic AII-stimulated redistribution of radioactivity between [^{3}H]phosphoinositide, [^{3}H]inositol and [^{3}H]phosphoinositol fractions of pre-labelled bovine zfr cells. Cells were pre-labelled for 6 hr with [^{3}H]inositol in the presence or absence of Mn^{2+} or ACh, as described. Cells were subsequently incubated for a further 2 hr in the presence or absence of AII (10^{-7} M) in fresh medium containing 10 mM Li^+. The distribution of radioactivity is shown as in Fig. 1. Values shown are the means ± SD of triplicate determinations from one representative experiment of three similar experiments.

achieved by subsequent challenge with AII (10^{-7} M) was not significantly different to that observed in control cells. Thus, the same steady state (see Discussion) was achieved on acute stimulation with AII, whether cells were pre-incubated with ACh or not. In addition, no significant difference in the phosphoinositol response to acute AII stimulation was observed between control and ACh pre-treated cells (c).

Pre-labelling cells with [^{3}H]inositol for 6 hr in the presence of Mn^{2+} resulted in an increased incorporation of label into the phosphoinositides (a) accompanied by a correspondingly reduced free inositol lablelling (b) (both $P < 0.05$). However, on subsequent acute (15 min) stimulation with AII (10^{-7} M), the incremental increases in the labelling of the phosphoinositides (a) and phosphoinositols (c), and the loss of [^{3}H]inositol (b) were not significantly different from those measured in cells labelled in the absence of Mn^{2+}.

Figure 2 shows the changes in the distribution of radioactivity between the three [^{3}H]inositol pools in cells chronically stimulated with AII (2 hr) following pre-incubation with ACh or Mn^{2+}, as above. Chronic AII stimulation resulted in a loss of radioactivity from the phosphoinositide and free [^{3}H]inositol pools (a,b), and a correspondingly large phosphoinositol response (c) (all $P < 0.05$). Although pre-labelling in the presence of ACh increased incorporation of [^{3}H]inositol into the phosphoinositides (a) ($P < 0.01$), the level to which phosphoinositide labelling and free [^{3}H]inositol levels dropped in response to subsequent chronic AII stimulation (a) was unaf-

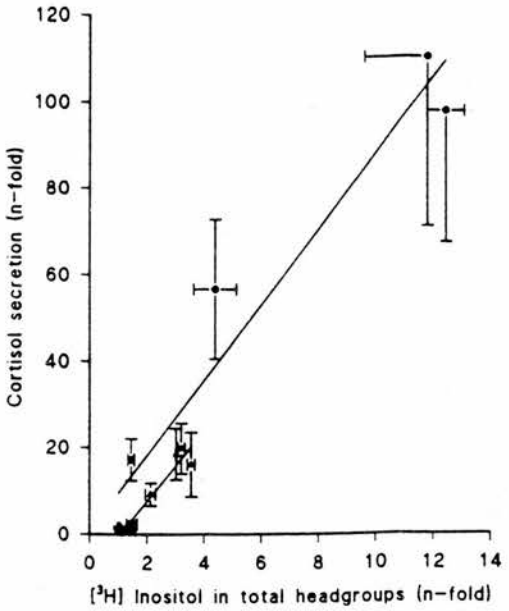

Fig. 3. Correlation between AII- and ACh-stimulated cortisol secretion and phosphoinositol production in primary cultures of bovine zfr cells. Cortisol secretion (over 1 hr) and phosphoinositol production (over 15 min) were measured in response to ACh (10^{-9}–10^{-3} M) (▲) and AII (10^{-11}–10^{-7} M) (●) as described. Results are expressed as *n*-fold stimulation ratios, and show the linear relationship between cortisol secretion and phosphoinositidase C activation (as measured by the accumulation of aqueous-labelled phosphoinositol headgroups) for both agonists. Regression analysis of the data gave correlation coefficients (*r*) of 0.972 and 0.964 for ACh and AII, respectively. The gradients of the regression lines were not significantly different (8.38 ± 1.07 and 8.34 ± 1.2, respectively). Values shown are the means ± SEM of data from 3–6 experiments, each performed in triplicate.

fected by ACh pre-stimulation, as was the magnitude of the phosphoinositol response to AII (c). In contrast, when cells pre-labelled in the presence of Mn^{2+} were subsequently stimulated chronically (2 hr) with AII in the presence of Li^+, the elevated level of phosphoinositide labelling in Mn^{2+} pre-treated cells (a) was decreased on AII stimulation by the same amount, but not down to the same level, as cells not pre-treated with Mn^{2+}. In addition, the magnitude of the phosphoinositol response to AII was reduced following Mn^{2+} pre-stimulation compared with that seen in both control and ACh pre-treated cells ($P < 0.01$).

We have demonstrated previously that both ACh and AII produce dose-dependent increases in cortisol secretion in bovine zfr cells, accompanied by a corresponding activation of phosphoinositidase C with similar dose dependency [6]. Figure 3 illustrates the correlation between phosphoinositidase C activation (as measured by the accumulation of water-soluble [^{3}H]phosphoinositol headgroups over 15 min, in cells pre-labelled to steady state) and the cortisol response (over 60 min) for both AII (10^{-11}–10^{-7} M) and ACh (10^{-9}–10^{-3} M). A strong correlation was revealed between these two responses for each agonist (correlation coeffecients: ACh, $r = 0.972$ and AII, $r = 0.964$; both $P < 0.01$). Moreover, the gradients of the regression lines for AII and ACh were not significantly different (8.32 ± 1.21 and 8.34 ± 1.07, respectively).

DISCUSSION

The effects of AII and ACh on phosphoinositide resynthesis and subsequent breakdown were examined in cells pre-labelled with [^{3}H]inositol for 6 hr. We have previously shown that cells labelled for this length of time contain large reserves of [^{3}H]inositol in the cytosol but only incorporate small amounts of label into the phosphoinositides [13]. Because cells are not labelled to steady state, resynthesis of phosphoinositides (as a consequence of agonist-stimulated phosphoinositide turnover) increases labelling of the phosphoinositide pool. If stimulation is also carried out in the presence of Li^+ to prevent recycling of the phosphoinositols into inositol, AII (10^{-7} M) stimulates incorporation to a new steady state within 15 min. This new steady state is maintained for up to 30 min of stimulation. After 30 min, cytosolic [^{3}H]inositol becomes depleted so that newly synthesized lipid is catabolized without resynthesis. Thus, at stimulation times longer than 30 min, breakdown of this newly labelled lipid can be observed [13].

Figure 1 shows the effect of ACh and Mn^{2+} pre-stimulation on the subsequent redistribution of [^{3}H]inositol in response to acute AII stimulation. Although ACh preincubation increased phosphoinositide labelling, the maximal incorporation of label into the phosphoinositide pool in response to AII was not significantly different between control and ACh pre-treated cells. This is consistent with the stimulation by ACh of incorporation of [^{3}H]-inositol into the same hormone-sensitive phosphoinositide pool as that utilized by AII (i.e. if ACh had stimulated labelling of a different pool than that acted upon by AII, then the label incorporation into phosphoinositides would be greater with ACh preincubation than without). Consistent with this conclusion, the magnitude of the phosphoinositol response to acute AII stimulation in the presence of Li^{2+} was unaffected by ACh pre-stimulation during labelling.

We have also studied the effects of pre-labelling cells in the presence of 1 mM Mn^{2+}. Treatment with Mn^{2+} has been shown in other cells to stimulate labelling by both phosphatidylinositol synthase activity and inositol headgroup exchange [15–17]. However, this process appears to stimulate the labelling of a hormone-insensitive pool of inositol phospholipid [18]. Pre-labelling zfr cells with [^{3}H]-inositol in the presence of Mn^{2+} increased the incorporation of label into the phosphoinositides and reduced free inositol labelling. However, unlike ACh pre-stimulation, the subsequent acute AII stimulation resulted in a significant increase in the maximum phosphoinositide labelling, though the incremental increase in phosphoinositide and phosphoinositol labelling, and the loss of [^{3}H]inositol were not significantly different from those measured

in control cells (labelled in the absence of Mn^{2+}). Thus, these findings are consistent with Mn^{2+}, unlike ACh, promoting the labelling of a phosphoinositide pool distinct from that acted on by AII.

ACh pre-stimulation had no effect on the level to which phosphoinositide labelling and free [^{3}H]-inositol dropped in response to subsequent chronic AII stimulation, and did not alter the magnitude of the accompanying phosphinositol response (Fig. 2). Thus, the lipid synthesized in response to the ACh pre-stimulation was accessible to the phosphoinositidase C activated by AII stimulation and therefore must have been in the same hormone sensitive pool. In contrast, when cells pre-labelled in the presence of Mn^{2+} were subsequently stimulated chronically with AII in the presence of Li^+, the pool of phosphoinositide labelled in response to Mn^{2+} during the pre-incubation remained inaccessible to the AII-stimulated phosphoinositidase C. Cells pre-treated with Mn^{2+} showed the same incremental decrease in phosphoinositide labelling in response to chronic AII stimulation as did control cells. These findings also support stimulation by Mn^{2+} of the labelling of a hormone-insensitive pool of phosphoinositide.

It could be argued that the hormone-insensitive pool of [^{3}H]inositol phospholipid labelled in the presence of Mn^{2+} represents labelling of 3-phosphorylated phospholipids. However, HPLC analysis of the phosphoinositols formed in response to AII stimulation in bovine zfr cells indicates that 3-phosphate derivatives of the phosphoinositides are not formed [19]. Even if 3-phosphorylated phosphoinositides are not substrates for phosphoinositidase activity [20], FPLC and HPLC studies of the deacylated phosphoinositides from bovine zfr cells have also failed to detect multiple isomeric forms of phosphatidylinositol bisphosphate (I.M.B., unpublished data).

We have shown previously that ACh and AII activate a hormone-sensitive phosphoinositidase C, and stimulate the mobilization of a common intracellular pool of calcium. The data presented here reveal that both ACh and AII also lead to the breakdown and resynthesis of a common hormone-sensitive pool of inositol phospholipid. A similar situation appears to occur in rat zona glomerulosa cells for vasopressin and AII [21], and in WRK-1 cells stimulated with bradykinin and vasopressin [22].

We also demonstrate (Fig. 3) that the gradients of the cortisol versus [^{3}H]inositol headgroup responses for ACh and AII were not significantly different in cells labelled to steady state. Such a finding is consistent with both ACh and AII acting through a common mechanism and common pool of phosphoinositide to promote steroidogenesis. The coupling of two or more receptors to a common pool of phosphoinositide in the same cell has implications with respect to the possible modulation of the effects of one agonist by the other. Homologous sensitization or desensitization to either agonist must occur through changes at the level of the receptor, whereas heterologous desensitization would be expected to result from changes in the phosphoinositidase C–G protein complex.

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ngiotensin II Stimulates Growth and Steroidogenesis in ona Fasciculata/Reticularis Cells from Bovine Adrenal ortex via the AT_1 Receptor Subtype*

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STRACT

Primary cultures of bovine adrenocortical zona fasciculata/reticu- s (zfr) cells responded to angiotensin II (AII) with a dose-dependent rease in [^{3}H]thymidine incorporation into DNA. The effect was ximal at 100 nmol/liter AII, and was dose dependently inhibited by ^{1},ala^8)-AII (saralasin) and DuP753, but not by PD123177. Both -stimulated cortisol secretion and phosphoinositidase C activity e also inhibited by saralasin and DuP753, but not by PD123177. armacological analysis of the antagonism of AII-stimulated cortisol secretion by saralasin and DuP753 produced pA_2 values of 8.79 and 7.02, respectively. Whereas the pA_2 for saralasin agreed closely with previous measurements in other systems, DuP753 was at least one order of magnitude less potent in inhibiting the action of AII in bovine zfr cells compared to previous measurements in rabbit vascular smooth muscle. We conclude that the steroidogenic and mitogenic effects of AII in bovine zfr cells are mediated by the AT_1 receptor. (*Endocrinology* **132:** 2206–2212, 1993)

THE octapeptide angiotensin II (AII) exerts a wide variety of physiological responses in many target tissues in- ding vascular smooth muscle, heart, kidney, brain, and enal (see Refs. 1, 2 for review). In addition to its well aracterized steroidogenic effect on adrenal zona glomeru- a (zg) cells (3), AII also stimulates cortisol secretion from vine zona fasciculata/reticularis (zfr) cells through the ivation of hormone sensitive phosphoinositidase C (PIC) viewed in Ref. 4).

AII is a potent mitogen in several tissues including vascular d cardiac smooth muscle, and this effect has implicated in the pathogenesis of hypertension (5). The mitogenic ect of AII in adrenocortical zg cells is well documented; stimulates growth of rat zg cells (6), and activation of the in angiotensin system is accompanied by hypertrophy of rat zg (7). The trophic effects of AII in inner adrenocortical nes are less well understood. Growth of rat zona fasciculata) cells has been reported in response to AII (8), and ereas increased [^{3}H]thymidine incorporation into the DNA unpurified bovine adrenocortical cells in culture has been monstrated (9, 10), the possible mitogenic effects of AII in rified bovine zfr cells have not been studied.

ncreasing evidence suggests that bovine zfr cells in culture y be a more relevant model than other species for human enocortical inner zone cells. Rat (11) and sheep (12) zfr cells respond to AII with increased phosphoinositide turnover and inositol phosphate production, yet neither species show an accompanying steroidogenic response to AII, even though ovine and bovine zfr cells possess a similar number of specific AII receptors (12). Human zf cells, by contrast, do secrete cortisol in response to AII (13), and while the mechanism of this stimulation remains unclear, involvement of the calcium messenger system is suggested by the steroidogenic effects of the calcium ionophore A23187 and the phorbol ester phorbol 12-myristate 13-acetate in these cells (14). Furthermore, AII stimulates the breakdown of membrane phosphatidylinositol-4,5 bisphosphate in cultured human zfr cells (authors' unpublished observations).

Whereas previous work has generally failed to support an involvement of AII in stimulating human inner zone cells *in vivo* (15, 16), AII did produce dose-dependent increases in plasma cortisol in normal human subjects when infused simultaneously over a low constant infusion of ACTH (17), and may therefore be important in the regulation of inner zone function in man.

Using selective nonpeptide antagonists, two subtypes of AII receptors have been identified, termed AT_1 (sensitive to DuP753) and AT_2 (sensitive to PD123177 and analogs) (18). Inhibition of ^{125}I-AII binding by these antagonists has shown the presence of both AT_1 and AT_2 binding sites in many tissues including adrenal, uterus, brain, and aorta (19, 20), and marked species differences in the distribution of the binding sites between tissues has been reported (20).

Whereas both AT_1 and AT_2 binding sites have been identified in the adrenal cortex of rat, human (19, 21), rabbit (22), and monkey (20), unpurified bovine adrenocortical cells in culture appear to contain only the AT_1 binding site (23), and in cultured bovine zg cells, both AII-stimulated aldosterone

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cretion and inhibition of ACTH-stimulated cAMP production are mediated by the AT_1 receptor (23).

There have been no studies specifically addressing growth fects of AII in bovine inner zone cells, or attempts to haracterize the particular receptor subtype present in these lls. In the present study, the ability of AII to stimulate [^{3}H] ymidine incorporation into bovine zfr cells in primary ulture is reported. The antagonist sensitivity of the mitoenic, steroidogeneic, and second messenger responses to AII ere investigated in this purified zfr cell preparation. Adrecortical AII receptors have not previously been characterized by Schild analysis. This analysis provides antagonist A_2 values ($-\log_{10}$ antagonist dissociation constant) through easurement of the potency of an antagonist in inhibiting e functional response of a tissue to an agonist (24). The alue of pA_2 is unique for a given antagonist acting at a ven receptor subtype, irrespective of the agonist used to icit the response, and differences in pA_2 measurements etween tissues can therefore yield valuable information garding receptor heterogeneity. Accordingly, we also report A_2 values for both the peptide antagonist (sar^1,ala^8)-AII aralasin), and for the AT_1 receptor antagonist DuP753 for e first time in adrenocortical cells.

Materials and Methods

The source of all cell culture materials is described in Clyne *et al.* (25). ntrolled Process Serum Replacement no. 5 (CPSR5), PBS tablets, onidet P-40, and (sar^1,ala^8)-angiotensin II (saralasin) were from the gma Chemical Co. (Poole, Dorset, UK). Dulbecco's Modified Eagle's edium (DMEM) was obtained from ICN/Flow (High Wycombe, UK). *yo*[^{3}H]inositol, *methyl*[^{3}H]thymidine, and cortisol tracer [cortisol-3-(*O*-rboxymethyl)oximino-2(-[^{125}I]iodohistamine)] were purchased from nersham International (Aylesbury, Bucks, UK). Anion exchange resin as obtained from Bio-Rad Laboratories (Watford, Herts, UK), and ntillation fluid and vials were from Canberra Packard (Pangbourne, rkshire UK) AII (asp^1-val^5) was the Medical Research Council standard /15. Dup753 (also known as losartan potassium and MK 954) (2-*n*-tyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-methyl]imidazole, potassium salt) and PD123177 [1-(4-amino-3-ethylphenyl)methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1H-imizo[4,5-c]pyridine-6-carboxylic acid-2HCl] were kind gifts from Dr. land D. Smith (DuPont, Wilmington, DE).

ll preparation and culture

Primary cultures of bovine adrenal zfr cells were prepared as debed previously (26). Cells were dispensed into 12-well culture dishes -mm wells) at a density of 330,000 cells/ml·well (165,000 cells/ml·ll for [^{3}H]thymidine uptake studies) in a medium of Ham's F10 with % (vol/vol) CPSR5, penicillin (50 IU/ml), streptomycin (50 μg/ml), d amphotericin B (2.5 μg/ml). After 24 h, medium was replaced with her 0.5 ml identical fresh medium, 0.5 ml fresh medium supplemented th 10 μCi/ml [^{3}H]inositol or, for [^{3}H]thymidine uptake studies, 0.5 ml MEM containing antibiotics as above, but without added CPSR5; in e absence of serum, cells are known to be growth arrested (9).

easurement of [^{3}H]thymidine uptake

Forty eight hours after initial plating, growth arrested cells were shed twice with 1 ml DMEM and left under 0.5 ml DMEM in the esence of the agonists and antagonists indicated. After 15 h cells were ain washed with 2 × 1 ml DMEM, and medium replaced with 0.5 ml MEM containing fresh agonists/antagonists, supplemented with [^{3}H] midine (5 μCi/ml). Stimulation was allowed to proceed for 6 h, after which cells were washed with PBS (2 × 1 ml), and taken up in 1 ml PBS containing 0.1% (vol/vol) Nonidet P-40 (PBS/N). Well contents were filtered through Whatman GF/C glass fiber filters with a 1 ml PBS/N wash, and the filters washed with a further 2 × 5 ml PBS/N. Filters were then soaked in ethanol and air dried. Finally, [^{3}H]thymidine counts were measured using 3 ml scintillation fluid on a Canberra Packard 1900CA scintillation counter.

Measurement of PIC activation

The activation of PIC in cells prelabeled with [^{3}H]inositol for 48 h was determined by measuring the hormone-stimulated generation of total [^{3}H]inositol phosphates in the presence of 10 mmol/liter LiCl for 15 min as described previously (27). Aqueous [^{3}H]inositol phosphates were extracted and separated from free [^{3}H]inositol on columns of anion exchange resin according to Bird *et al.* (28).

Measurement of cortisol secretion

Forty eight hours after initial plating, cells were washed twice with 1 ml Earl's Balanced Salt solution (EBS) with added BSA and glucose [0.2 and 0.1% (wt/vol), respectively] (EBSBG) and left under 0.4 ml. After 5-min preincubation at 37 C, antagonists were added in a volume of 50 μl EBSBG. AII (dissolved in EBSBG, 50 μl) was added after a further 5 min. Stimulation was allowed to proceed for 60 min, after which the overlying medium was removed and stored at −20 C before cortisol RIA (29).

Data analysis

Unless otherwise indicated, values shown represent the mean ± SEM of triplicate determinations from three separate experiments from different cell preparations. Significance between values was assessed using Student's *t* test. Inhibition curves were fitted to a sigmoid power model on an IBM computer using Fig P (Biosoft) for calculation of ID_{50} values, and those ID_{50} values reported in the text are the mean of three such determinations from separate cell preparations. For analysis of dose-response data, curves were tested for parallelism according to Kenakin (30). Where the slope of Schild regression lines (24) did not differ significantly from unity, data were fitted to the best regression line of slope unity for calculation of pA_2 values (31).

Results

Effect of AII on [^{3}H]thymidine incorporation in bovine zfr cells

Figure 1 shows the effect of increasing concentrations of AII on DNA synthesis (as measured by the uptake of [^{3}H] thymidine over 6 h) in growth arrested bovine zfr cells. AII stimulated [^{3}H]thymidine incorporation into DNA in a dose-dependent manner, with half-maximal effect at 0.25 nmol/liter, and maximal effect at 100 nmol/liter, at which concentration [^{3}H]thymidine uptake was stimulated 2.32-fold (n = 3). Significant stimulation ($P < 0.05$) was observed at AII concentrations of 10 nmol/liter-1 μmol/liter.

Effect of nonselective and selective AII antagonists on AII-stimulated [^{3}H]thymidine incorporation

To determine which AII receptor subtype is responsible for the mitogenic action of AII in bovine zfr cells, the ability of the nonselective antagonist saralasin and the selective nonpeptide antagonists DuP753 and PD123177 to inhibit AII-stimulated [^{3}H]thymidine incorporation was investigated. As shown in Fig. 2, saralasin (10 nmol/liter-10 μmol/liter) completely inhibited the mitogenic effect of AII (10

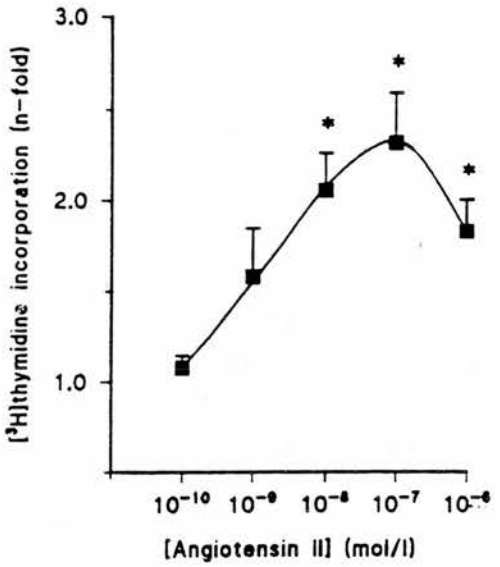


FIG. 1. Effect of increasing concentrations of AII on [^{3}H]thymidine incorporation into DNA in cultured bovine zfr cells. Growth arrested bovine adrenal zfr cells were challenged with AII at the concentrations indicated for 21 h. [^{3}H]Thymidine (5 μCi/ml) was present for the last 6 h, adding fresh AII at the same concentration, as described. Values are expressed as a stimulation ratio relative to the basal (unstimulated) incorporation, and represent the mean ± SEM of triplicate determinations from three separate experiments. Mean basal [^{3}H]thymidine incorporation was 9330 ± 1552 dpm/well. Significant stimulation at $P < 0.05$ relative to basal is indicated (*).

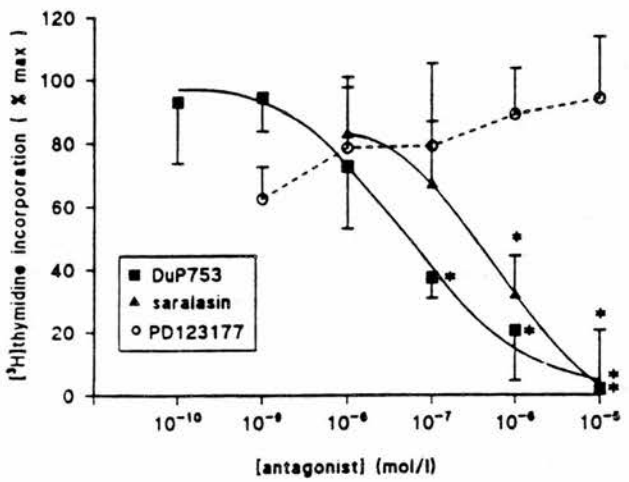


FIG. 2. Effects saralasin, DuP753, and PD123177 on AII-stimulated [^{3}H]thymidine incorporation in cultured bovine zfr cells. Growth arrested cells were challenged with AII (10 nmol/liter) in the presence or absence of (■) DuP753, (▲) saralasin or (○) PD123177 at the concentrations indicated for 21 h. [^{3}H]thymidine (5 μCi/ml) was present for the last 6 h of this period. Values are expressed as a percentage of the control (AII, 10 nmol/liter) incorporation. Each point is the mean ± SEM of triplicate determinations from three separate cell preparations. Significant inhibition relative to the control AII response is indicated $P < 0.05$ (*) and $P < 0.01$ (**).

mol/liter), producing significant inhibition at 1 μmol/liter ($P < 0.05$). The ID_{50} for saralasin was 315 nmol/liter (range, 10–780 nmol/liter).

The AT_1 receptor antagonist DuP753 also inhibited AII (10 mol/liter)-stimulated [^{3}H]thymidine incorporation in a dose-dependent manner (Fig. 2), reaching significance at 0.1 μmol/liter, and producing complete inhibition at 10 μmol/liter. Dup753 was slightly more potent than saralasin (ID_{50} = 50 nmol/liter, range 12–130 nmol/liter). In contrast, the AT_2-selective antagonist PD123177 did not significantly affect the magnitude of the mitogenic response to AII at concentrations up to 10 μmol/liter PD123177.

Saralasin, DuP753 and PD123177 had no intrinsic effect on [^{3}H]thymidine incorporation at concentrations up to 10 μmol/liter (data not shown).

Effect of saralasin, DuP753, and PD123177 on AII-stimulated cortisol secretion and [^{3}H]inositol phosphate production

Previous studies have shown that AII-stimulated cortisol secretion from cultured bovine zfr cells is mediated through activation of PIC. Cells prelabeled with [^{3}H]inositol for 42 h (to steady state) secrete cortisol and produce aqueous [^{3}H] phosphoinositols (in the presence of Li^+) in response to AII with similar dose dependency (28). The effects of saralasin and DuP753 on AII-stimulated cortisol secretion and [^{3}H] inositol phosphate production are shown in Fig. 3, a and b, respectively. Both saralasin and DuP753 completely inhibited cortisol secretion in response to a maximal dose of AII (10 nmol/liter) with ID_{50} values of 120 nmol/liter (range 62–150 nmol/liter) and 3.1 μmol/liter (range 0.2–7.9 μmol/liter, respectively (Fig. 3a). Complete inhibition was achieved at 10 μmol/liter saralasin and 100 μmol/liter DuP753.

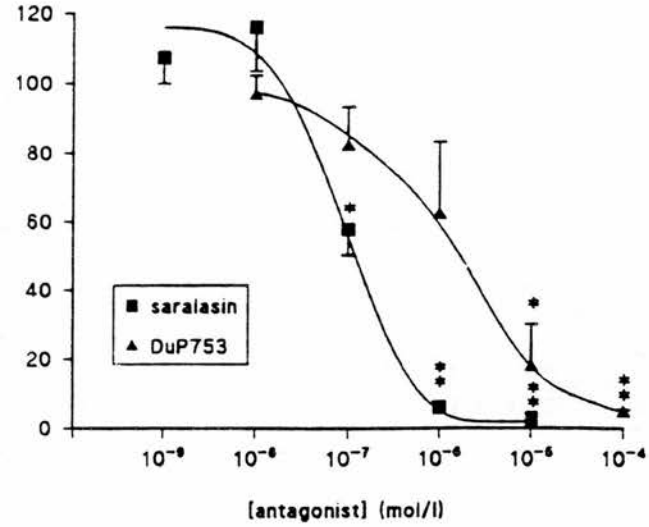
Saralasin and DuP753 also completely inhibited the production of [^{3}H]inositol phosphates in response to AII (10 nmol/liter) (Fig. 3b) with ID_{50} values of 51 nmol/liter (range 19–69 nmol/liter) and 0.49 μmol/liter (range 0.3–1.1 μmol/liter), respectively.

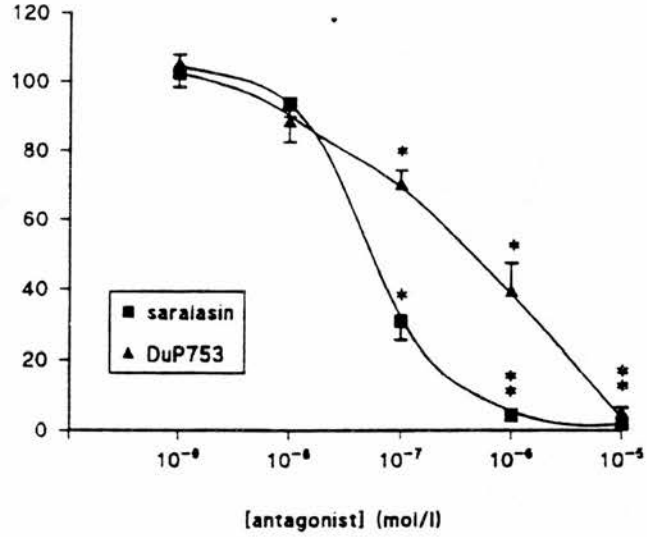
Neither saralasin nor DuP753 had any effect on either basal cortisol secretion or [^{3}H]inositol phosphate production at concentrations up to 100 μmol/liter antagonist (data not shown).

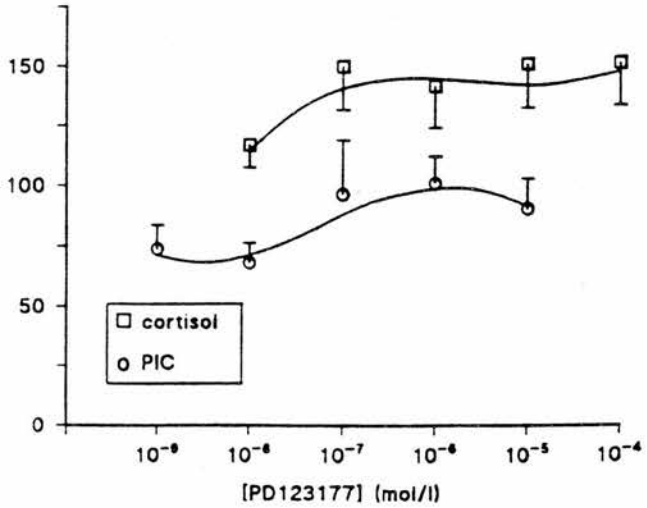
Figure 3c shows the effects of the AT_2-selective antagonist PD123177 on AII-stimulated cortisol secretion and [^{3}H]inositol phosphate production. PD123177 did not significantly alter the magnitude of the steroidogenic or second messenger responses to AII (10 nmol/liter) at concentrations up to 0.1 mmol/liter PD123177. PD123177 was also without effect on either basal cortisol secretion or [^{3}H]inositol phosphate production at concentrations up to 0.1 mmol/liter (data not shown).

Effect of saralasin and DuP753 on the cortisol dose response curve to AII

To further characterize the receptor responsible for AII-stimulated cortisol secretion, dose-response curves to AII were set up in the presence of increasing concentrations of saralasin and DuP753. Representative experiments are shown in Fig. 4 a and b, respectively. Both saralasin (10 nmol/liter-0.33 μmol/liter, Fig. 4a) and DuP753 (0.33 μmol/liter-10 μmol/liter, Fig. 4b) produced parallel shifts of the AII dose-response curve, without affecting the maximal response attainable by AII, consistent with competitive antagonism.







3. Effects of saralasin, DuP753, and PD123177 on AII-stimulated sol secretion and [^{3}H]inositol phosphate production by zfr cells. a, were incubated for 1 h with AII (10 nmol/liter) or AII (10 nmol/ the presence of (■) saralasin or (▲) DuP753 at the concentrations ated. Cortisol secretion is shown *vs.* antagonist concentration. b,

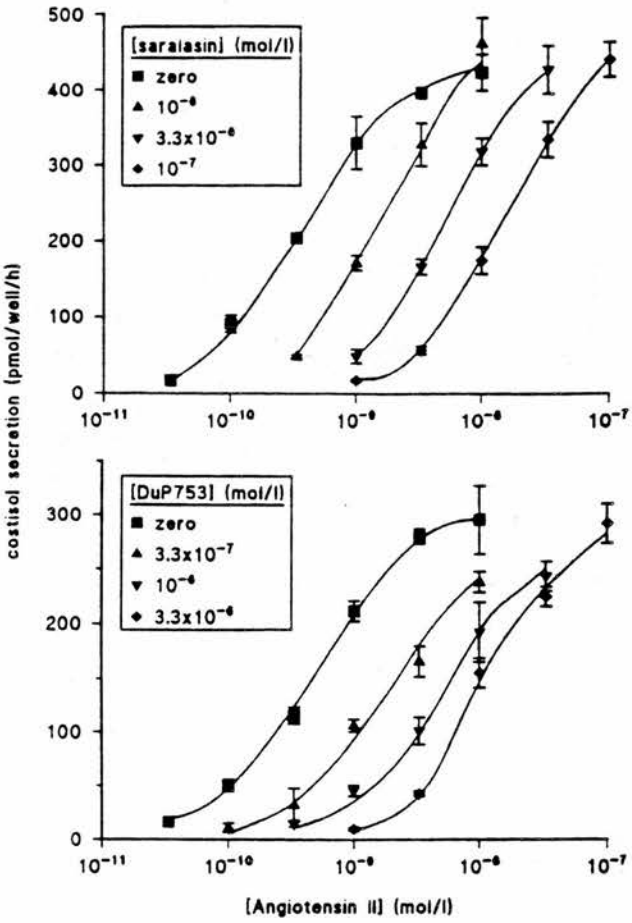


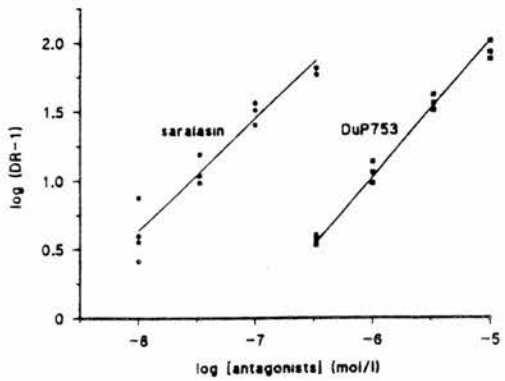
FIG. 4. Representative experiments showing dose response curves for the secretion of cortisol stimulated by AII alone (■) and by AII in the presence of increasing concentrations of (*upper panel*) saralasin (*lower panel*), DuP753. Values shown are the mean ± SD of triplicate determinations. Similar results were obtained from four separate cell preparations for each antagonist.

The dose ratio (DR) for each antagonist concentration was obtained and Schild analysis [$\log_{10}$ (DR-1) *vs.* $\log_{10}$ [antagonist]] performed to calculate antagonist pA_2 values (Fig. 5). The results of this analysis are summarized in Table 1.

Discussion

The observation that AII is a potent mitogen in several tissues including bovine (9, 10) and rat (8) adrenocortical

[^{3}H]Inositol prelabeled cells were challenged with AII (10 nmol/liter) in the presence or absence of (■) saralasin or (▲) DuP753 for 15 min (in the presence of LiCl, 10 mmol/liter). Total aqueous [^{3}H]inositol phosphate production is shown *vs.* antagonist concentration. c, (□) cortisol secretion and (○) [^{3}H]inositol phosphate production in response to AII (10 nmol/liter) were determined in the presence or absence of PD123177 at the concentrations shown. All values are expressed as a percentage of the control (AII, 10 nmol/liter) response, and each point is the mean ± SEM of triplicate determinations from three separate cell preparations. Mean basal cortisol secretion was 5.37 ± 0.83 pmol/well·h, and secretion stimulated by AII (10 nmol/liter) was 491.4 ± 53.1 pmol/well·h. Mean basal and mean AII (10 nmol/liter)-stimulated [^{3}H]inositol phosphate production were 4774 ± 286 and 59,581 ± 8,960 dpm/well/15 min, respectively. Significant inhibition relative to the control AII response is indicated at $P < 0.05$ (*) and $P < 0.01$ (**).



G. 5. Schild regressions, least squares fit of $\log_{10}$ (DR-1) *vs.* $\log_{10}$ ntagonist] where DR = dose ratio. Schild regressions for saralasin) and DuP753 (■) are shown. Cumulative data from eight separate ll preparations.

ABLE 1. Comparison of experimental and published pA_2 values r saralasin and DuP753

Antagonist	Experimental pA_2	95% CL	slope	95% CL	Published pA_2
Saralasin	8.79	8.41–9.17	0.81	0.66–0.96	9.01[42]
DuP753	7.02	6.89–7.15	0.93	0.85–1.02	8.48[42,47]

95% CL, 95% confidence limits.

ells led us to investigate the possible mitogenic effects of II in the bovine zfr, which is known to exhibit mitogenic sponses to other peptide growth factors (32). Studies of the itogenic effect of AII in bovine adrenocortical cells have enerally failed to determine whether the site of action is stricted to the zg, or also involves the zf. Neither has the articular AII receptor subtype mediating the steroidogenic id/or mitogenic effects of AII in bovine zfr cells been vestigated.

In the present study, we demonstrate that AII stimulates H]thymidine incorporation into purified bovine zfr cells ith a potency and efficacy comparable to that previously ported for unpurified bovine adrenocortical cells in culture). This response was completely inhibited both by saralasin nd DuP753, but not by PD123177 suggesting that, as in the onal adrenocortical AC1 cell line (33), the mitogenic action AII in purified bovine zfr cells is mediated by the AT_1 ceptor. This receptor subtype is known to mediate growth sponses to AII in other cell types, including rat aortic nooth muscle cells (34).

The mechanism of AII-induced mitogenesis in adrenocor-cal cells is unknown at present, although a recent report nplicates both activation of protein kinase C and production 12-lipoxygenase products in the mitogenic action of AII 3), in addition to possible protein-tyrosine phosphorylation 5), and activation of phosphoinositidase C (36).

We have also characterized the receptor subtype respon-ble for the steroidogenic and second messenger responses AII in bovine zfr cells. Cortisol secretion in response to II was completely inhibited by the nonselective AII receptor ntagonist saralasin, and by the AT_1-selective antagonist uP753, indicating the involvement of the AT_1 receptor in the steroidogenic response to AII. This same receptor subtype is known to mediate aldosterone secretion from cultured bovine zg cells (23), and dispersed rat adrenal capsular cells (37). As expected, the AT_2-selective antagonist PD123177 was without effect.

AII-stimulated cortisol secretion from bovine zfr cells is mediated by activation of PIC (28). The formation of [^{3}H] phosphoinositols in response to AII was also inhibited both by saralasin and DuP753, but not by PD123177, again consistent with the involvement of the AT_1 receptor. A similar inhibition of AII-stimulated PIC activity by DuP753 has been reported in clone 9 cells from rat liver (38). In addition, COS-7 cells transfected with the AII-1 receptor cDNA from bovine adrenal zg or rat aortic smooth muscle responded to AII with an increase in phosphoinositol production (39, 40), whereas no such response was observed in R3T3 cells, which express AT_2 receptors only (41).

It is of interest that saralasin was around one order of magnitude more potent than DuP753 in inhibiting both AII-stimulated cortisol secretion and [^{3}H]inositol phosphate production, but was clearly less potent than DuP753 in inhibiting the mitogenic response to AII. Whereas it is tempting to speculate that the mitogenic and steroidogenic effects of AII may therefore be mediated by different DuP753-sensitive receptors, this may be a result of the different incubation conditions used for measurement of these responses. Cortisol secretion and [^{3}H]inositol phosphate production were measured acutely, whereas for measurement of [^{3}H]thymidine uptake, cells were exposed to AII and/or antagonists for a total of 21 h. If saralasin were degraded at a faster rate than the nonpeptide antagonist DuP753 over this time period, then the relative potency of saralasin would be erroneously low.

To further characterize the antagonism of AII-stimulated cortisol secretion by saralasin and DuP753, analysis by Arunlakshana and Schild (24) was performed to calculate antagonist pA_2 values. Both antagonists shifted the AII concentration-response curve to the right, in a parallel manner, without depressing the maximum response attainable by AII, consistent with competitive antagonism. The extrapolated pA_2 for saralasin (8.79) agreed well with previous reports in other systems (42). Peptide analogs of AII have previously been reported to exhibit partial agonist properties, and saralasin has also been described as a noncompetitive antagonist of AII-stimulated responses both *in vitro* (43, 44) and *in vivo* (45), as indicated by its nonparallel shifts of the AII dose response curve and depression of the maximum response. No such activity was observed in the present study for the antagonism of AII-stimulated cortisol secretion by saralasin. It is possible that the doses of saralasin employed (10 nmol/liter-0.33 μmol/liter) were insufficient for partial agonism to be detected. However, depression of the maximum response to AII-induced contraction of rabbit aorta by saralasin is apparent at 3 nmol/liter saralasin (42). As reported by others (43) the slope of the Schild regression was significantly lower than unity. This may be indicative of an agonist uptake process which would result in underestimation of the dose ratio and produce a Schild slope less than unity. Indeed

uptake of AII by cultured bovine adrenocortical cells has been reported, with AII internalization occurring with a half-life of 10 min (46). A further possibility is that saralasin acts in a noncompetitive manner as reported by Wong *et al.* (43). In tissues with a large receptor reserve this would result in parallel shifts of the dose response curve without depression of the maximal response at low antagonist concentrations.

Consistent with previous observations (42, 47), DuP753 acted as a classical competitive antagonist of AII-stimulated cortisol secretion, producing parallel shifts of the AII dose-response curve with no depression of the maximum response. The resultant Schild regression was linear with a slope not significantly different from unity. However, the observed pA_2 for DuP753 (7.02) was about 30 times lower than that previously reported for the antagonism of AII-mediated contraction of rabbit aorta ($pA_2 = 8.48$). Although differences in assay conditions might be expected to lead to a difference in pA_2 values for the same receptor, pA_2 measurements are generally in good agreement between laboratories (48). The magnitude of the difference in pA_2 values for DuP753 in bovine zfr cells and smooth muscle was therefore unexpected. One possible explanation is that DuP753 recognizes two distinct subtypes of AT_1 receptor in vascular smooth muscle and adrenal cortex, respectively. This hypothesis would accord with previous data from both functional and structural studies; although AII acts via the AT_1 receptor in both bovine adrenocortical and rat aortic smooth muscle cells (23, 47), $(sar)_5$-AII, and $(pro)_3$-AII were weak agonists/partial antagonists of AII-stimulated steroidogenesis in bovine adrenocortical cells but acted as full agonists of AII-stimulated responses in rat aortic smooth muscle cells (49). Furthermore, two subtypes of the rat AT_1 receptor (AT_{1A} and AT_{1B}) have been cloned (50–52). Although no differences in the ligand binding characteristics between these subtypes have yet been detected, AT_{1A} and AT_{1B} receptors appear to be preferentially expressed between vascular smooth muscle and adrenal cortex respectively, and their expression subject to differential regulation by steroids (53).

In conclusion, we have demonstrated that AII is mitogenic for bovine adrenocortical inner zone cells in primary culture, and that both the acute steroidogenic and long-term mitogenic responses to AII are mediated by the AT_1 receptor. Although the present data are insufficient to explain the unusually low pA_2 for DuP753 obtained in bovine adrenocortical cells, the possibility that distinct subtypes of AT_1 receptor are recognized by DuP753 is currently under investigation.

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325

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THE M_3 MUSCARINIC RECEPTOR MEDIATES ACETYLCHOLINE-INDUCED CORTISOL SECRETION FROM BOVINE ADRENOCORTICAL ZONA FASCICULATA/RETICULARIS CELLS.

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Abstract—In order to characterize the receptor subtype mediating acetylcholine (ACh)-induced cortisol secretion from purified bovine adrenocortical zona fasciculata/reticularis cells in primary culture, the potencies of a range of selective muscarinic antagonists of ACh-induced steroidogenesis were assessed by Schild analysis. Basal secretion of cortisol was 10.2 ± 1.4 pmol/well/30 min. ACh stimulated a dose-dependent increase in cortisol secretion and was maximally effective at 10^{-5} M, at which concentration cortisol secretion was 143.4 ± 12.9 pmol/well/30 min. Hexahydro-sila-difenidol and para-fluoro-hexahydro-sila-difenidol were potent competitive antagonists of ACh-stimulated cortisol secretion, with pA_2 values of 8.68 ± 0.28 and 7.96 ± 0.29, respectively. Pirenzepine (pA_2 = 6.95 ± 0.28) and methoctramine (pA_2 = 6.06 ± 0.27) were relatively weak competitive antagonists. The pA_2 values determined in this study are characteristic of the M_3 muscarinic receptor, and we conclude that this receptor subtype mediates ACh-induced cortisol secretion from bovine zona fasciculata/reticularis cells.

Key words:

Stimulation of adrenocortical steroid secretion in response to ACh§ was first demonstrated in the isolated perfused bovine adrenal gland [1]. ACh has since been reported to stimulate steroidogenesis from the perfused rat and amphibian adrenal [2, 3] and from cells isolated from both the bovine zg and zfr [4–6]. It is significant that direct cholinergic innervation of the adrenal cortex has been observed in several species including sheep [7] and man [8]. Furthermore, a possible role for ACh in regulating adrenocortical steroidogenesis in man has been proposed [9].

While no effect of ACh on cAMP levels has been demonstrated in adrenocortical inner zone cells [5, 6], ACh clearly stimulates PtdIns turnover. Dose-dependent increases in $^{32}P_i$ labelling of PtdIns occur in response to ACh [10], and in purified bovine zfr cells, ACh stimulates the formation of inositol tris- and bis-phosphates, the time course of appearance of which is consistent with the activation of a polyphosphoinositide specific phosphoinositidase C [6].

Where studied, stimulation of steroidogenesis and second messenger formation by ACh have been sensitive to muscarinic, but not nicotinic antagonists [3, 5, 6, 10, 11]. However, the muscarinic receptor subtype involved in the steroidogenic response to ACh has not been established.

Of the five muscarinic receptor subtypes which have been cloned [12–15], the m1, m2 and m3 receptors appear to correlate with the pharmacologically defined M_1, M_2 and M_3 subtypes [16]. Since none of the muscarinic antagonists currently available is more than 10-fold selective for a given receptor subtype, it is necessary to obtain affinity profiles for a range of antagonists in order to classify muscarinic receptor subtypes definitively. Such antagonists include pirenzepine ($M_1 > M_2 = M_3$), methoctramine ($M_2 > M_1 > M_3$), HHSD ($M_1 = M_3 > M_2$) and p-FHHSD ($M_3 > M_2 = M_1$) [17–20]. Recently a fourth receptor subtype has been pharmacologically identified (M_4) exhibiting high affinity for the M_1- and M_2-selective antagonists pirenzepine and AF-DX-116 [21, 22]. This putative M_4 receptor subtype can be distinguished from the M_1 receptor by its high affinity for himbacine and methoctramine [21, 23], and appears to correspond to the muscarinic m4 gene product expressed in chick heart and brain [24].

Preliminary observations indicated that stimulation of steroidogenesis from primary cultures of bovine zfr cells by ACh may occur through the M_3 muscarinic receptor [6]. The present study was undertaken to definitively characterise the receptor subtype involved in this response, by determining the affinities of a range of selective antagonists of ACh-stimulated cortisol secretion by Schild analysis [25, 26].

MATERIALS AND METHODS

Materials. The source of all cell culture and

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§ Abbreviations: ACh, acetylcholine; zg, zona glomerulosa; zfr, zona fasciculata/reticularis; EBS, Earle's Balanced Salts solution; HHSD, hexahydro-sila-difenidol; p-FHHSD, para-fluoro-hexahydro-sila-difenidol; PtdIns, phosphatidylinositol.

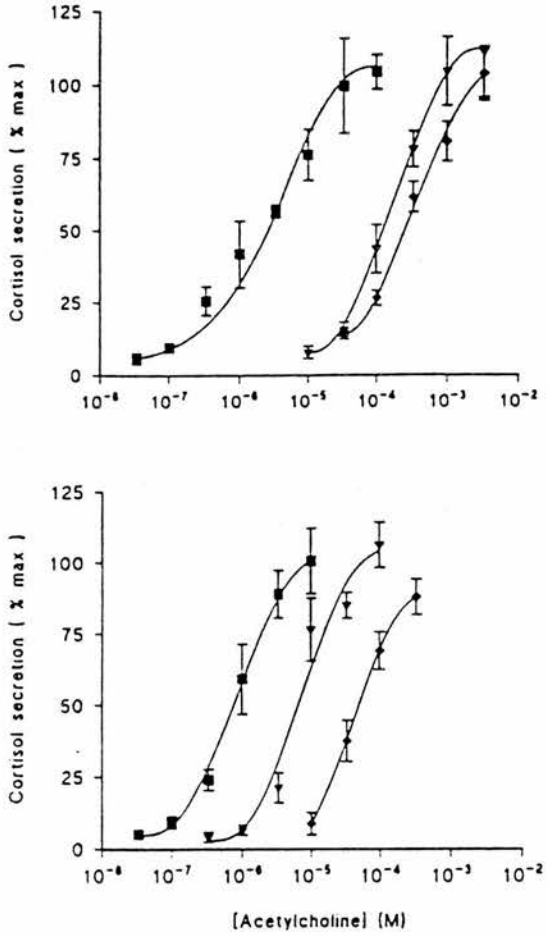

Fig. 1. Dose–response curves for the secretion of cortisol by cultured bovine zfr cells produced on stimulation with ACh alone (■) and in the presence of (upper panel) pirenzepine (▼) 3.3×10^{-9}M, (◆) 10^{-5}M; (lower panel) methoctramine (▼) 10^{-5}M, (◆) 10^{-4}M. Data points are the means ± SD of triplicate determinations from one representative experiment for each antagonist.

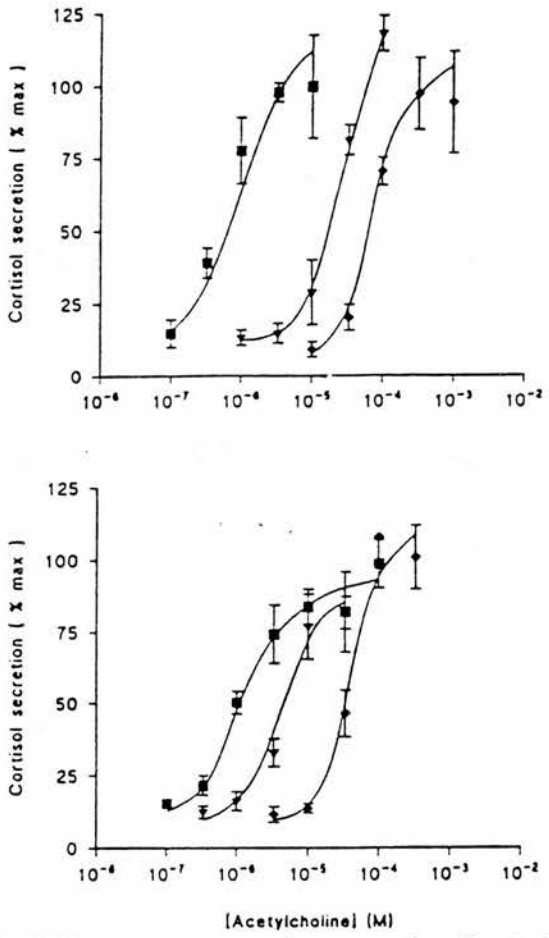

Fig. 2. Dose–response curves for the secretion of cortisol by cultured bovine zfr cells produced on stimulation with ACh alone (■) and in the presence of (upper panel) HHSD (▼) 3.3×10^{-6}M, (◆) 10^{-7}M; (lower panel) p-FHHSD (▼) 3.3×10^{-8}M, (◆) 3.3×10^{-7}M. Data points are the means ± SD of triplicate determinations from one representative experiment for each antagonist.

radioimmunoassay materials is described in Ref. 27. Controlled Process Serum Replacement No. 5 (CPSR-5), acetylcholine and pirenzepine were from the Sigma Chemical Co. (Poole, U.K.). HHSD hydrochloride and p-FHHSD hydrochloride were kind gifts from Drs G. Lambrecht, E. Mutschler and R. Tacke of the Johann-Wolfgang-Gothe University (Frankfurt am Main, Germany). *N,N'*-Bis[6-(2-methoxybenzly)amino]hexyl - 1.8 - octane - diamine tetrahydrochloride (methoctramine) was a generous gift from Prof. Dr C. Melchiorre, Universita' Degli Studi di Bologna, (Italy).

Cell culture and stimulation. Bovine adrenal glands were obtained from freshly slaughtered 1–2 year old steers (Gorgie Abattoir, Edinburgh, U.K.). Purified zfr cell suspensions were prepared by the collagenase digestion and column filtration procedure described previously [28], producing a zfr cell preparation essentially free from glomerulosa and medullary cell contamination [28]. Cells were cultured at 37° under 5% Co_2 in 12-well culture dishes (25 mm diameter wells) at 333,000 cells/mL, in 1 mL of Ham's F10 medium containing 10% (v/v) CPSR-5, penicillin (50 IU/mL), streptomycin (50 μg/mL) and amphotericin B (2.5 μg/mL) (F10 growth medium). After 24 hr the overlying medium was replaced with 0.5 mL of identical fresh medium.

Experiments were performed 48 hr after initial plating, at which time cells showed peak responsiveness to ACh [29]. F10 growth medium was removed and cells washed twice with 1 mL of EBS solution with added BSA and glucose [0.2 and 0.1% (w/v) respectively] (EBSBG). Cells were pre-incubated for 5 min under 0.4 mL EBSBG after which ACh and/or antagonists, dissolved in EBSBG, were added to the cells to a final volume of 0.5 mL. Stimulation was allowed to proceed for 30 min at 37° after which the overlying medium was removed and stored at −20° prior to cortisol radioimmunoassay as described previously [28].

Data analysis. In order to obtain estimates of

dose–response curve parameters, data sets were fitted by iterative non-linear least squares regression analysis using the software package Fig P (Biosoft) on an IBM computer, allowing the maximum and half-maximum responses to be calculated. Dose–response curves were tested for parallelism according to Kenakin [30], and antagonist pA_2 values estimated by the method of Arunlakshana and Schild [26]:

$$\log_{10}(r - 1) = n\log_{10}[B] + pK_B$$

in which r is the dose ratio produced by an antagonist of concentration [B], n is the Schild plot slope and pK_B is the antagonist affinity constant. Where n was found to be not significantly different from unity, pK_B was estimated with n constrained to unity [31].

RESULTS

A series of concentration–response curves to ACh were set up in the presence of increasing doses of the muscarinic antagonists pirenzepine, methoctramine, HHSD and p-FHHSD. Representative experiments are shown in Figs 1 and 2. Six such experiments were carried out for each antagonist in order to determine pA_2 values. Basal cortisol secretion was 10.2 ± 1.4 pmol/well/30 min (mean ± SEM, N = 24 experiments). The dose–response curve for ACh fell in the concentration range 10^{-7}–10^{-5}M, and the ED_{50} for ACh-stimulated cortisol secretion was 1.1×10^{-6}M (range $0.4–7.2 \times 10^{-6}$M). Maximal stimulation of cortisol secretion occurred at 1×10^{-5}M ACh, at which concentration cortisol secretion was 143.4 ± 12.9 pmol/well/30 min (mean ± SEM, N = 24).

The effect of each of the antagonists studied was to shift the log dose–response curve to the right. None of the antagonists had any effect on the maximal response attainable by ACh or the slope of the dose–response curve, consistent with competitive antagonism (Figs 1 and 2). None of the antagonists had any intrinsic effect on cortisol secretion at any of the concentrations employed (up to 1×10^{-5}M HHSD, 1×10^{-6}M p-FHHSD, 1×10^{-4}M pirenzepine and methoctramine) (data not shown).

For estimation of antagonist affinities, the dose ratio (r) for each concentration of antagonist was obtained and Schild plots [26] constructed (Fig. 3). The estimated antagonist pA_2 values and slope of the Schild regressions are shown in Table 1.

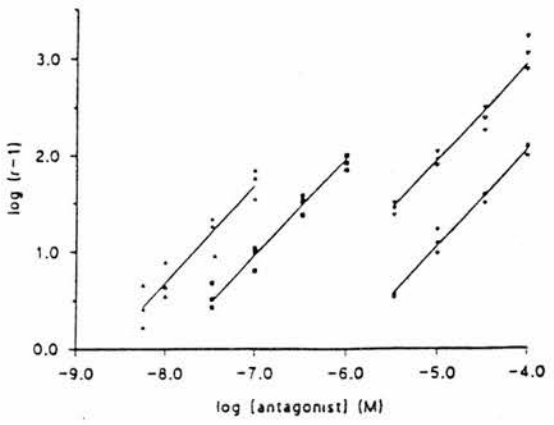

Fig. 3. Schild analysis $\log_{10}$ (dose-ratio (r)-1) vs $\log_{10}$[antagonist]) of the antagonism by (▲) HHSD, (■) p-FHHSD, (▼) pirenzepine and (◆) methoctramine of ACh-stimulated cortisol secretion from bovine zfr cells. Cumulative data from six separate cell preparations for each antagonist (two concentrations of antagonist per experiment).

DISCUSSION

Bovine adrenocortical ACh receptors have previously been characterized as muscarinic both in the outer zg and in the inner zfr [4, 5]. Walker *et al.* [6] reported the effects of a number of cholinergic agonists and antagonists on cortisol secretion from zfr cells, and obtained preliminary evidence for the presence of an M_3 receptor. However, definitive investigation of the muscarinic receptor subtypes present in the adrenal cortex has not been carried out, either by radioligand binding or by functional analysis, in any species. The present study establishes that ACh-stimulated cortisol secretion from bovine adrenocortical inner zone cells occurs through activation of the M_3 muscarinic receptor, as discussed below.

The use of Schild analysis for calculation of antagonist pA_2 values is valid only for competitive antagonists. The antagonists employed in this study produced parallel shifts of the log dose–response curve to ACh without depressing the maximum response to ACh, and produced linear Schild regressions with slopes not significantly different from unity, consistent with competitive antagonism. The rank potency order (HHSD > p-FHHSD > pirenzepine > methoctramine) is characteristic of that of the M_3 muscarinic receptor: the relatively low potencies of pirenzepine ($pA_2 = 6.95$) and methoctramine ($pA_2 = 6.06$) indicate that the steroidogenic response to ACh is not mediated by M_1 or M_2 receptors respectively (see Ref. 32 for review), while the high pA_2 values for HHSD ($pA_2 = 8.68$) and p-FHHSD ($pA_2 = 7.96$) are characteristic of M_3 receptor function [19, 20].

Recently a fourth muscarinic receptor subtype has been pharmacologically characterized which appears to correspond to the m4 gene product [21]. While the bovine adrenal medulla has been shown to express mRNA transcript exclusively for the m4 receptor, a weak signal for the m4 receptor mRNA was also detected in the cortex [33]. Although the putative M_4 receptor subtype exhibits high affinity for the M_2-selective compounds HHSD and p-FHHSD [21], it also has moderate to high affinity for methoctramine similar to that of pirenzepine in both ligand binding and functional assays [21, 23]. The low pA_2 for methoctramine observed in bovine zfr cells argues against any significant involvement of the M_4 receptor in ACh—stimulated cortisol secretion.

While the observed pA_2 values for pirenzepine, methoctramine and p-FHHSD correlate well with the known values at M_3 receptors in other systems

Table 1. Comparison of experimental pA_2 values for muscarinic antagonists in bovine zfr cells with previously published values

Antagonist	$pA_2 \pm$ SEM	slope ± SEM	Published pA_2				Reference
			M_1	M_2	M_3	M_4	
Pizenzepine	6.95 ± 0.28	1.06 ± 0.06	8.0–8.3	6.5–6.8	6.7–7.0	7.2–7.7	[21, 23, 38–43]
Methoctramine	6.06 ± 0.27	1.01 ± 0.04	6.8–7.6	7.6–8.3*	5.9–6.9	7.6–7.8	[21, 23, 38, 39, 42, 43]
HHSD	8.68 ± 0.28	1.02 ± 0.10	7.9–8.7	6.5–6.9	7.6–8.5	7.6–7.9*	[21, 38, 40, 44]
p-FHHSD	7.96 ± 0.29	0.95 ± 0.05	6.7–7.2	6.0–6.1	7.6–8.0	7.8	[16, 38–40, 42, 43]

* $-\log_{10}K_i$.

(Table 1), that for HHSD was slightly higher in zfr cells than would be expected at any muscarinic receptor. While this value is not inconsistent with an M_3 receptor classification, since HHSD exhibits highest affinity at M_1/M_3 receptors, it does require comment. A similarly high value (8.49) has been reported previously in ovine detrusser smooth muscle [34]. Although carbachol was used as the agonist, this would not be expected to affect the apparent antagonist affinity, since measurements of pA_2 are independent of the agonist used to elicit the functional response [30]. It seems unlikely that this discrepancy is due to inadequate antagonist equilibration time, since the existence of a non-equilibrium steady-state would be expected to produce a Schild slope greater than unity [30]: the Schild slopes for each antagonist in the present study were not significantly different from unity. It is possible that the unusually high pA_2 for HHSD reported in the present study reflects a species-dependent heterogeneity of M_3 receptors arising from differences in receptor or G protein structure. The precise reasons for this anomaly are unclear at present, and require further investigation.

The presence of the M_3 muscarinic receptor is consistent with the previously reported effects of ACh in bovine zfr cells: (i) stimulation of phosphoinositidase C activity [6, 10], the second messenger system associated with the cloned m1, m3 and m5 gene products, and with native M_1 and M_3 cholinoceptors; (ii) the lack of effect of ACh on cAMP levels in adrenocortical cells [5, 6]; (iii) the inability of the M_1-selective agonist McN-A-343 to stimulate cortisol secretion from bovine zfr cells [6].

Although the significance of ACh in controlling adrenocortical steroidogenesis remains to be established, a number of observations clearly support an *in vivo* function. ACh stimulates cortisol secretion from both the isolated perfused bovine adrenal gland and from freshly isolated bovine zfr cells [1, 6]. In addition, acetylcholinesterase—positive innervation of the adrenal cortex has been detected in many species including sheep [7] and man [8]. While administration of muscarinic agonists to humans had no intrinsic effect on plasma steroid concentration, the plasma aldosterone response to angiotensin II infusion was inhibited by atropine [9]. Observations such as these suggest that cholinergic mechanisms may exert a modulatory effect on the actions of other adrenocortical agonists. Such modulatory effects could occur either through interactions between ACh and other agonists at the level of the steroidogenic cells, as have been reported to occur in the amphibian adrenal [35, 36], or through effects of ACh on adrenocortical blood flow: in conscious calves, infusion of ACh resulted in a decrease in adrenocortical vascular resistance and an enhancement of the steroidogenic effect of adrenocorticotropin [37]. Thus, while data presented here and elsewhere clearly indicate a direct steroidogenic action of ACh on purified adrenocortical cells, it seems likely that ACh may also enhance agonist-stimulated steroidogenesis *in vivo* by increasing adrenal blood flow, thereby increasing the delivery of such agonists to the steroidogenic tissue.

In conclusion, the antagonist affinity profiles observed in the present study most closely resemble those of the M_3 muscarinic receptor, and we propose that this receptor subtype mediates ACh-stimulated cortisol secretion from bovine zfr cells. The functional significance of this muscarinic cholinergic element in the regulation of adrenocortical steroidogenesis *in vivo* warrants further investigation.

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